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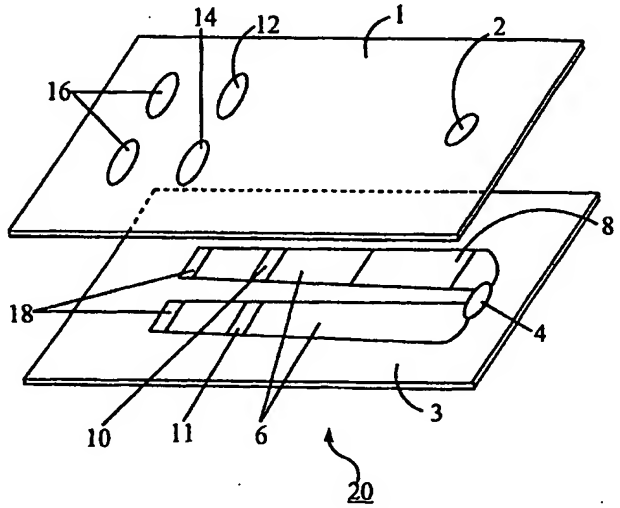

(57) Abstract

The present invention relates to rapid methods for the detection of ischemic states and to kits for use in such methods. Provided for is a rapid method of testing for and quantifying ischemia based upon methods of detecting and quantifying the existence of an alteration of the serum protein albumin which occurs following an ischemic event; methods for detecting and quantifying this alteration include evaluating and quantifying the cobalt binding capacity of circulating albumin, analysis and measurement of the ability of serum albumin to bind exogenous cobalt, detection and measurement of the presence of endogenous copper in a purified albumin sample and use of an immunological assay specific to the altered form of serum albumin which occurs following an ischemic event. Also taught by the present invention is the detection and measurement of an ischemic event by measuring albumin N-terminal derivatives that arise following an ischemic event, including truncated albumin species lacking one to four N-terminal amino acids or albumin with an acetylated N-terminal Asp residue.

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TESTS FOR THE RAPID EVALUATION OF ISCHEMIC STATES AND KITS

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to rapid methods for the detection of ischemic states and to kits for use in such methods. More particularly, the invention relates to the measurement of a bound specific transition element to human serum albumin or the measurement of albumin N-terminal derivatives to determine the presence or absence of ischemia.

2. Discussion of the Background

Ischemia is the leading cause of illness and disability in the world. Ischemia is a deficiency of oxygen in a part of the body causing metabolic changes, usually temporary, which can be due to a constriction or an obstruction in the blood vessel supplying that part. The two most common forms of ischemia are cardiovascular and cerebrovascular. Cardiovascular ischemia, in which the body's capacity to provide oxygen to the heart is diminished, is the leading cause of illness and death in the United States. Cerebral ischemia is a precursor to cerebrovascular accident (stroke) which is the third leading cause of death in the United States.

The continuum of ischemic disease includes five conditions: (1) elevated blood levels of cholesterol and other blood lipids; (2) subsequent narrowing of the arteries; (3) reduced blood flow to a body organ (as a result of arterial narrowing); (4) cellular damage to an organ caused by a lack of oxygen; (5) death of organ tissue caused by sustained oxygen deprivation. Stages three through five are collectively referred to as "ischemic disease," while stages one and two are considered its precursors.

Together, cardiovascular and cerebrovascular disease accounted for 954,720 deaths in the U.S. in 1994. Furthermore, more than 20% of the population has some form of cardiovascular disease. In 1998, as many as 1.5 million Americans will have a new or recurrent heart attack, and about 33% of them will die. Additionally, as many as 3 to 4 million Americans suffer from what is referred to as "silent ischemia." This is a condition where no clinical symptoms of ischemic heart disease are present.

There is currently a pressing need for the development and utilization of blood tests able to detect injury to the heart muscle and coronary arteries. Successful

treatment of cardiac events depends largely on detecting and reacting to the presence of cardiac ischemia in time to minimize damage. Cardiac enzymes, specifically the creatine kinase isoenzyme (CK-MB), and cardiac markers, specifically the Troponin I and T biochemical markers, are utilized for diagnosing heart muscle injury. However, these enzymes and markers are incapable of detecting the existence of an ischemic state in a patient prior to myocardial infarction and resulting cell necrosis (death of cell). Additionally, these enzymes and markers do not show a measurable increase until several hours after an ischemic event. For instance, CK-MB, the earlier evident of the two, does not shows a measurable increase above normal in a person's blood test until about four to six hours after the beginning of a heart attack and does not reach peak blood level until about 18 hours after such an event. Thus, the primary shortcoming of using cardiac markers for diagnosis of ischemic states is that these markers are only detectable after heart tissue has been irreversibly damaged.

There currently are no tests available which allow diagnosis of the existence of ischemia in patients prior to tissue necrosis. A pressing requirement for emergency medicine physicians who treat chest pain and stroke symptoms is for a diagnostic test that would enable them to definitively "rule out" myocardial infarction, stroke, and other emergent forms of ischemia. A need exists for a method for immediate and rapid distinction between ischemic and non-ischemic events, particularly in patients undergoing acute cardiac-type symptoms. The present invention provides such a means.

A broader array of diagnostic tests are available for diagnosis of ischemia in patients with non-acute symptoms. The EKG exercise stress test is commonly used as an initial screen for cardiac ischemia, but is limited by its accuracy rates of only 25-50%. Coronary angiography, an invasive procedure that detects narrowing in the arteries with 90-95% accuracy, is also utilized. Another commonly used diagnostic test is the thallium exercise stress test, which requires injection of radioactive dye and serial tests conducted four hours apart. The present invention, however, has the advantage over the known methods of diagnosis in that it provides equivalent or better accuracy at far lower costs and decreased risk and inconvenience to the patient. The

present invention provides specificity and sensitivity levels of 75-95%, which are far more accurate than the EKG exercise stress test and comparable in accuracy to current diagnostic standards. Furthermore, the present invention presents a significant time advantage and is cheaper than competing methods of diagnosis by a factor of at least 15 to 1.

It is known that immediately following an ischemic event, proteins (enzymes) are released into the blood. Well known proteins released after an ischemic heart event include creatine kinase (CK), serum glutamic oxalacetic transaminase (SGOT) and lactic dehydrogenase (LDH). One well known method of evaluating the occurrence of past ischemic heart events is the detection of these proteins in a patient's blood. U.S. Pat. No. 4,492,753 relates to a similar method of assessing the risk of future ischemic heart events. However, injured heart tissue releases proteins to the bloodstream after both ischemic and non-ischemic events. For instance, patients undergoing non-cardiac surgery may experience perioperative ischemia. Electrocardiograms of these patients show ST-segment shifts with an ischemic cause which are highly correlated with the incidence of postoperative adverse cardiac events. However, ST-segment shifts also occur in the absence of ischemia; therefore, electrocardiogram testing does not distinguish ischemic from non-ischemic events. The present invention provides a means for distinguishing perioperative ischemia from ischemia caused by, among other things, myocardial infarctions and progressive coronary artery disease.

SUMMARY OF THE INVENTION

The present need for rapid, immediate and continuous detection of ischemic states is met by the present invention. Specifically, the present invention provides for rapid methods of testing for the existence of and quantifying ischemia based upon methods of detecting and quantifying the existence of an alteration of the serum protein albumin which occurs following an ischemic event. Preferred methods of the present invention for detecting and quantifying this alteration include evaluating and quantifying the metal binding capacity of albumin, analysis and measurement of the ability of serum albumin to bind exogenous metal, detection and measurement of the presence of endogenous copper in a purified albumin sample, use of an immunological assay specific to albumin-metal complexes, and detection and measurement of albumin N-terminal derivatives that arise following an ischemic event. Also taught by the present invention is the use of the compound Asp-Ala-His-Lys-R, wherein R is any chemical group capable of being detected when bound to a metal ion that binds to the N-terminus of naturally occurring human albumin, for detection and quantitation of an ischemic event.

Advantages and embodiments of the invention include a method for ruling-out the existence of an ischemic state or event in a patient; a method for detecting the existence of asymptomatic ischemia; a method for evaluating patients with angina to rule-out the recent occurrence of an ischemic event; an immediate method for evaluation of patients suffering from chest pain to detect the recent occurrence of a myocardial infarction; a method for evaluation of patients suffering from stroke-like signs and symptoms to detect the occurrence of a stroke and to distinguish between the occurrence of an ischemic stroke and a hemorrhagic stroke; a rapid method for supplementing electrocardiographic results in determining the occurrence of true ischemic events; a method for detecting the occurrence of a true ischemic event in a patient undergoing surgery; a method for evaluating the progression of patients with known ischemic conditions; a method for comparing levels of ischemia in patients at rest and during exercise; a method for assessing the efficacy of an angioplasty procedure; a method for assessing the efficacy of thrombolytic drug therapy; a

method for assessing the patency of an in-situ coronary stent; and, a method for detecting in a pregnant woman the occurrence of placental insufficiency.

Additional advantages, applications, embodiments and variants of the invention are included in the Detailed Description of the Invention and Examples sections.

As used herein, the term "ischemic event," and "ischemic state" mean that the patient has experienced a local and/or temporary ischemia due to partial or total obstruction of the blood circulation to an organ. Additionally, the following abbreviations are utilized herein to refer to the following amino acids:

Amino acid	Three-letter abbreviation	Single-letter notation
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Asparagine or aspartic acid	Asx	B
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glutamine or glutamic acid	Glx	Z
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

A separate test method for ischemia was described by a common inventor in U.S. Patents Nos. 5,227,307 and 5,290,519 to Bar-Or et al., herein incorporated by reference in their entirety. Also incorporated herein in their entireties by reference are the following commonly assigned applications: U.S. Serial No. 09/165,926, filed October 2, 1998; U.S. Serial No. 09/165,581, filed October 2, 1998; and U.S. Serial No. 60/115,392, filed January 11, 1999.

BRIEF DESCRIPTION OF THE FIGURES

Figs. 1-3 illustrate kits useful in carrying out the derivative embodiment of the subject invention.

Fig. 4 shows selected regions of the ^{1}H-NMR spectra (500 MHz, 10% D_2O in H_2O, 300K) showing the Ala resonances (Ala-2 and Ala-8) of the octapeptide (Asp-Ala-His-Lys-Ser-Glu-Val-Ala) (a) free of any metal, with a Lys-4 methylene resonance appearing between the doublets, (b) with 0.5 equiv. of $NiCl_2$ added, (c) with 1.0 equiv. of $NiCl_2$ added, (d) with 0.5 equiv. of $CoCl_2$ added, and (e) with 1.0 equiv. of $CoCl_2$ added.

Figs. 5A and 5B are ultraviolet spectra for non-acetylated Pep-12 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys) and acetylated Pep-12, respectively.

Figs. 6A and 6B are ultraviolet spectra for non-acetylated Pep-12 and acetylated Pep-12 each with $CoCl_2$, respectively.

Fig. 7 provides spectral analysis of five solutions of increasing proportions of acetylated Pep-12 to non-acetylated Pep-12 with effect on cobalt binding as reflected by a shift in absorbance from 220 to 230.

Figs. 8A and 8B are U.V. spectra of Pep-12 and acetylated Pep-12, respectively, mixed first with $CuCl_2$ and then with $CoCl_2$.

Fig. 9 is the U.V. spectra of acetylated Pep-8 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala) which did not shift upon addition of cobalt.

Figs. 10A-D are the ^{1}H-NMR spectra of Peptide 1 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys) which shows the methyl signals of the two Ala residues at

positions 2 and 8 as titrated by $NiCl_2$. Fig. 10A is Peptide 1 at pH 2.55, while 10B is at pH 7.33. Fig. 10C is the spectra at pH 7.30 with 0.3 equiv. $NiCl_2$, and Fig. 10D is pH 7.33 at ~ 1 equiv. $NiCl_2$.

Figs. 11A-D are the ^{1}H-NMR spectra of Peptide 1 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys) which shows the methyl signals of the two Ala residues at positions 2 and 8 as titrated by $CoCl_2$. Fig. 11A is Peptide 1 at pH 2.56, while 11B is at pH 7.45. Fig. 11C is the spectra at pH 7.11 with ~0.5 equiv. $CoCl_2$, and Fig. 11D is pH 7.68 at ~ 1 equiv. $CoCl_2$.

Figs. 12A-D are the ^{1}H-NMR spectra of Peptide 1 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys) which shows the methyl signals of the two Ala residues at positions 2 and 8 as titrated by $CuSO_4$. Fig. 12A is Peptide 1 at pH 2.56, while 12B is at pH 7.54. Fig. 12C is the spectra at pH 7.24 with ~0.5 equiv. $CuSO_4$, and Fig. 12D is pH 7.27 at ~ 1 equiv. $CuSO_4$.

Figs. 13A-D are the ^{1}H-NMR spectra of Peptide 2, which is the acetylated-Asp version of Peptide 1. Fig. 13A is Peptide 2 at pH 2.63. Fig. 13B is Peptide 2 at pH 7.36. Fig. 13C is Peptide 2 at pH 7.09 with ~0.5 equiv. $NiCl_2$. Fig. 13D is Peptide 2 at pH 7.20 with ~1 equiv. $NiCl_2$.

Figs. 14A-E are the ^{1}H-NMR spectra of Peptide3 (Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys). Fig. 14A is Peptide 3 at pH 2.83. Fig. 14B is Peptide 3 at pH 7.15. Fig. 14C is Peptide 3 at pH 7.28 with ~ 0.13 equiv. $NiCl_2$. Fig. 14D is Peptide 3 at pH 7.80 with ~0.25 equiv. $NiCl_2$. Fig. 14E is Peptide 3 at pH 8.30 with ~0.50 equiv. $NiCl_2$.

Figs. 15A-D are the ^{1}H-NMR spectra of Peptide 4 (His-Lys-Ser-Glu-Val-ala-His-Arg-Phe-Lys). Fig. 15A is Peptide 4 at pH 2.72. Fig. 15B is Peptide 4 at pH 7.30. Fig. 15C is Peptide 4 at pH 8.30 with ~0.5 equiv. $NiCl_2$. Fig. 15D is Peptide 4 at pH 8.10 with ~1 equiv. $NiCl_2$.

Figs. 16A-D are the ^{1}H-NMR spectra of Peptide 5 (Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys). Fig. 16A is Peptide 5 at pH 2.90. Fig. 16B is Peptide 5 at pH 7.19.

Fig. 16C is Peptide 5 at pH1.02 with ~0.3 equiv. $NiCl_2$. Fig. 16D is Peptide 5 pH 7.02 with ~0.6 equiv. $NiCl_2$.

Figs. 17A-D are ^{1}H-NMR spectra of the N-terminal tetrapeptide, Asp-Ala-His-Lys. Fig. 17A is at pH 2.49. Fig. 17B is at pH7.44. Fig. 17C is at pH 7.42 with ~0.8 equiv. $NiCl_2$. Fig. 17D is at pH 7.80 with ~1 equiv. $NiCl_2$.

Figs. 18A-C are ^{1}H-NMR spectra of the N-terminal tetrapeptide with $CoCl_2$. Fig. 18A is at pH 7.44. Fig. 18B is at pH 7.23 with ~0.3 equiv. $CoCl_2$. Fig. 18C is at pH 7.33 with ~0.8 equiv. $CoCl_2$.

Figs. 19A-C are ^{1}H-NMR spectra of the N-terminal tetrapeptide with $CuSO_4$. Fig. 19A is at pH 7.31. Fig. 19B is at pH 7.26 with ~0.5 equiv. $CuSO_4$. Fig. 19C is at pH 7.32 with ~ 1.0 equiv. $CuSO_4$.

DETAILED DESCRIPTION OF THE INVENTION

A number of terms used herein have the following definitions.

"Albumin-metal complex" or "metal-albumin complex" means the complex of a divalent cation, including but not limited to copper, cobalt and nickel, to the N-terminus of naturally-occurring albumin.

"Albumin N-terminus" refers to that portion of naturally-occurring albumin constituting comprising at least the four N-terminal amino acids, i.e., Asp-Ala-His-Lys.

"Albumin N-terminal derivatives" refers to those species of albumin that are altered or truncated at the N-terminus as a result of an ischemic event. Specifically, the derivatives include those albumin species lacking 4, 3, 2 and 1 N-terminal amino acid, as well as a full-length albumin that is acetylated at its terminal Asp residue. Albumin -terminal derivatives cannot form albumin-metal complexes and may be found in the blood of ischemic patients. Full-length, naturally-occurring albumin is set forth is SEQ. ID. NO. 1. Acetylated-Asp albumin is set forth in SEQ. ID. NO. 2.

"Antibody to an albumin-metal complex" is an antibody to the epitope formed of the metal and surrounding amino acids and/or their side chains.

"Derivative N-terminus" refers to the 4-12 amino acids at the N-terminus of albumin N-terminal derivatives, which may serve as an epitope in the generation of a monoclonal antibody.

"Endogenous copper" refers to copper present in a patient sample of albumin, i.e., not exogenously added during the diagnostic procedure.

"Excess quantity" of metal ion or "excess metal ion" refers to addition of an amount of metal ion that will substantially exceed the stoichiometrically available albumin metal ion binding sites such that substantially all naturally-occurring albumin is bound to metal ion at its N-terminus.

"Known value" as used herein means a clinically-derived cut-off value or a normal range, to which a measured patient value is compared so as to determine the occurrence or non-occurrence of an ischemic event.

"Naturally-occurring albumin" refers to albumin with an intact N-terminus (Asp-Ala-His-Lys-) that has not been acetylated.

"Purified albumin" or "purified albumin sample" refers to albumin that has been partially purified or purified to homogeneity. "Partially purified" means with increasing preference, at least 70%, 80%, 90% or 95% pure.

"Treadmill test" means a stress test to increase myocardial O_2 demand, while observing if a mismatch occurs between demand and supply by observing symptoms such as shortness of breath, chest pain, EKG, low blood pressure and the like.

While not being bound by any particular theory, it is believed that the present method works by taking advantage of alterations which occur to the albumin molecule, affecting the N-terminus of albumin during an ischemic ("oxygen-depletion") event. (Ischemia occurs when human tissue is deprived of oxygen due to insufficient blood flow.) A combination of two separate phenomena are believed to explain the mechanism by which the ischemia test of the present invention works. First, it is believed that the localized acidosis which occurs during an ischemic event generates free radicals which alter albumin's N-terminus; thus, by detecting and quantifying the existence of altered albumin, ischemia can be detected and quantified.

Second, the acidotic environment present during ischemia results in the release of bound copper (from ceruloplasmin and other copper-containing proteins) which is immediately taken up by albumin. The bound copper also alters the N-terminus of albumin. (Not only does the presence of the complexed copper effectively "alter" the N-terminus, the metal ion damages the protein structure on binding.) Thus, by detecting and quantifying the existence of altered albumin, and/or the copper-albumin complexes, ischemia can be detected and quantified.

The details of the first mechanism are believed to be as follows. In the event of an oxygen insufficiency, cells convert to anaerobic metabolism, which depletes ATP, resulting in localized acidosis and lowered pH, and causing a breakdown in the energy cycle (ATP cycle). Cellular pumps that keep calcium against the gradient are fueled by energy from the ATP cycle. With ATP depletion, the pumps cease to function and cause an influx of calcium into the cell. The excess intracellular calcium activates calcium-dependent proteases (calpain, calmodulin), which in turn cleave segments of xanthine dehydrogenase, transforming the segments into xanthine oxidase. The enzymes involved in this process are membrane-bound and exposed to the outside of the cell, and are thus in contact with circulating blood. Xanthine oxidase generates superoxide free radicals in the presence of hypoxanthine and oxygen. Superoxide dismutase dismutates the oxygen free radicals, turning them into hydrogen peroxide. In the presence of metals such as copper and iron which are found in blood, hydrogen peroxide causes hydroxyl free radicals to be formed. Hydroxyl free radicals in turn cause damage to cells and human tissue. One of the substances damaged by free radicals is the protein albumin, a circulating protein in human blood; specifically believed to be damaged is the N-terminus of albumin, resulting in the albumin N-terminal derivatives.

Human serum albumin is the most abundant protein in blood (40g/l) and the major protein produced by the liver. Many other body fluids also contain albumin. The main biological function of albumin is believed to be regulation of the colloidal osmotic pressure of blood. The amino acid and structure of human albumin have been determined. Specifically, human albumin is a single polypeptide chain consisting of

585 amino acids folded into three homologous domains with one free sulfhydryl group on residue # 34. The specific amino acid content of human albumin is:

Residues:	Asp	Asn	Thr	Ser	Glu	Gln	Pro	Gly	Ala	Cys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Trp	Arg
Number	39	15	30	22	60	23	25	12	63	35	39	6	8	61	18	30	16	58	1	23

In the first embodiment of the present invention, an excess of metal (e.g., cobalt) ions are introduced into a (purified) albumin sample obtained from a patient serum, plasma, fluid or tissue sample (this embodiment is hereafter referred to as the "excess metal embodiment"). In normal (non-ischemic) patients, cobalt will bind to one or more amino acid chains on the N-terminus of albumin. In ischemic patients, however, most likely due to the alteration of the binding site of the N-terminus, cobalt binding to albumin is reduced. Accordingly, the occurrence or non-occurrence of an ischemic state can be detected by the presence and quantity of bound or unbound cobalt. Measurement of cobalt can be conducted by atomic absorption, infrared spectroscopy, high-performance liquid chromatography ("HPLC") or other standard or non-standard methods, including radioactive immunoassay techniques.

The details of the second mechanism are believed to be as follows. Ceruloplasmin is a circulating protein which binds copper; approximately ninety-percent of the in vivo copper (copper is abundant in blood, with concentrations comparable to iron) will be bound to ceruloplasmin. The remainder is in other bound forms; almost no free copper exists in circulating blood. In acidic conditions and reduced oxygen conditions, such as happens during ischemia, ceruloplasmin releases some of its bound copper. The released copper is taken up by albumin. Copper and cobalt both bind to albumin at the same site within the N-terminus. Thus, the bound endogenous copper, present during ischemia, blocks cobalt from binding to albumin. The decrease in cobalt binding capacity of circulating albumin can be measured and quantified as a means for detecting and quantifying the presence of an ischemic event.

The excess metal embodiment of the present invention comprises a method for detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of: (a) contacting a biological sample containing albumin of

said patient with an excess quantity of a metal ion salt, said metal ion capable of binding to the N-terminus of naturally occurring human albumin, to form a mixture containing bound metal ions and unbound metal ions, (b) determining the amount of bound metal ions, and (c) correlating the amount of bound metal ions to a known value to determine the occurrence or non-occurrence of an ischemic event. In this method, said excess quantity of metal ion salt may comprise a predetermined quantity and the quantity of unbound metal ions may be detected to determine the amount of bound metal ions. Additionally, the compound selected from the group consisting of Asp-Ala-His-Lys-R, wherein R is any chemical group capable of being detected when bound to a metal capable of binding to the N-terminus of naturally occurring human albumin, may be utilized to facilitate detection.

This method uses samples of serum or plasma, or purified albumin. Preferred embodiments also include use of a metal ion salt comprising a salt of a transition metal ion of Groups 1b-7b or 8 of the Periodic Table of the elements, a metal selected from the group consisting of V, As, Co, Cu Sb, Cr, Mo, Mn, Ba, Zn, Ni, Hg, Cd, Fe, Pb, Au and Ag. Also preferred, is detection of the amount of bound metal ions (or, in the case where the excess quantity of metal ion salt is a predetermined quantity, detection of the quantity of unbound metal ions) by atomic absorption or atomic emission spectroscopy or immunological assay. These detection mechanisms are also preferred for determination of the quantity of the compound Asp-Ala-His-Lys-R which is complexed with the metal ion salt in order to detect the quantity of unbound metal ions. A preferred method for conducting said immunological assay is using an antibody specific to an antigen comprising the compound Asp-Ala-His-Lys-R, wherein R is said metal ion.

Where the metal employed in the above excess metal embodiment is nickel, another preferred detection method is nuclear magnetic resonance (NMR). It has been observed that addition of Ni ion gives a sharp diamagnetic ^{1}H-NMR spectrum for the resonances of the first three amino acids (Asp-Ala-His) of the albumin N-terminus octapeptide. While Co ion can also induce changes in the NMR spectrum of the first three amino acids of albumin, it induces paramagnetism at the binding site, resulting

in broadening of the resonances associated with the first three residues. Thus, the diamagnetic nature of the nickel complex makes it more amenable for NMR studies.

The excess metal embodiment of the present invention also includes a colorimetric method of detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of: (a) contacting a biological sample containing albumin of said patient with a predetermined excess quantity of a salt of a metal selected from the group consisting of V, As, Co, Cu, Sb, Cr, Mo, Mn, Ba, Zn, Ni, Hg, Cd, Fe, Pb, Au and Ag, to form a mixture containing bound metal ions and unbound metal ions, (b) contacting said mixture with an aqueous color forming compound solution to form a colored solution, wherein said compound is capable of forming color when bound to said unbound metal ion, (c) determining the color intensity of said colored solution to detect the presence of unbound metal ions to provide a measure of bound metal ions, and (d) correlating the amount of bound metal ions to a known value to determine the occurrence or non-occurrence of an ischemic event. Preferred embodiments of this method include the additional step of diluting said colored solution with an aqueous solution isosmotic with blood serum or plasma prior to step (c). Also preferred are: using ferrozine as the color forming compound, and, alternatively, using the compound Asp-Ala-His-Lys-R, wherein R is any group capable of forming color when bound to said metal ion as the aqueous color forming compound. Conducting steps (b) and (c) in a pH range of 7 to 9 is preferred. Further, conducting steps (b) and (c) using a spectrophotometer is preferred. Preferred samples in this method include serum, plasma, or purified albumin and a preferred metal ion salt is cobalt.

Another embodiment is based on the endogenous copper mechanism discussed above. This embodiment involves a method for detecting the occurrence or non-occurrence of an ischemic state in a patient comprising the steps of: (a) detecting the amount of endogenous copper ions present in a purified albumin sample of said patient, and (b) correlating the quantity of copper ions present with a known value to determine the occurrence or non-occurrence of an ischemic event. Preferred methods for detection of the amount of copper ions present in the purified albumin sample are

by atomic absorption, atomic emission spectroscopy and immunological assay. A preferred method of conducting said immunological assay uses an antibody specific to an antigen comprising the compound Asp-Ala-His-Lys-R, wherein R is copper. This embodiment is referred to as the endogenous copper method.

Another embodiment of the subject invention is also based on the first mechanism described above. The free radicals released during an ischemic event damage the N-terminus of albumin by causing the cleavage of up to four N-terminal amino acid residues, and possibly may induce acetylation of the N-terminus. The resulting albumin derivatives lack the capacity to bind to metal ions such as cobalt ion. In the subject embodiment, an ischemic event is diagnosed by detecting the albumin derivatives that cannot bind metal ion. For this reason, the subject embodiment is referred to herein as the "derivative embodiment."

As is reported in the Examples, albumin having an acetylated terminal Asp or lacking four, three, two or even one N-terminal amino acid have been found to lack the capacity to bind to cobalt ion. It has been observed that albumin derivatives lacking four, three, two or one N-terminal amino acids are present in the serum or patients with ischemia.

The derivative embodiment of the subject invention comprises a method of detecting or measuring an ischemic event in a patient by: (a) contacting a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives with an excess quantity of metal ion that binds to the N-terminus of naturally-occurring albumin, whereby albumin-metal complexes are formed; (b) partitioning the complexes from said derivatives, if any; (c) measuring at least one of said derivatives, if any; and (d) comparing said measured derivative to a known value, whereby the ischemic event may be detected or measured.

The derivative embodiment method can be practiced with a metal ion salt that is a salt of a transition metal ion of Groups 1b-7b or 8 of the Periodic Table of the Element. Preferably, the metal ion salt is a salt of a metal selected from the group consisting of V, As, Co, Sb, Cr, Mo, Mn, Ba, Zn, Ni, Hg, Cd, Fe, Pb, Au and Ag. Most preferred is that the metal ion is Ni or Co. The minimum incubation period for

metal ion and albumin is at least 4-5 minutes, and preferably 10 minutes, i.e., an amount of time sufficient for equilibrium to be reached. It is also preferred that heparin be added to the sample prior to the addition of the excess quantity of metal ion.

The partitioning step of the derivative embodiment method can be carried out in two ways. It can be effected by having the excess metal ion of step (a) bound to a solid support such that the resulting albumin-metal complexes are retained on the solid support, permitting the elution separation of the albumin N-terminal derivatives. Alternatively, a solution of excess metal ion can be added to the patient sample, permitting the albumin-metal complexes to form, and the partitioning can be effected by contacting the complexes with antibodies to the metal-albumin complex that are bound to a solid support.

Thus, in one aspect, the derivative embodiment involves a method comprising: (a) contacting a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives with an excess quantity of a metal ion bound to a solid support, whereby the metal ion binds to the N-terminus of naturally-occurring albumin, forming metal-albumin complexes; (b) separating the complexes from said derivatives, if any; (c) measuring at least one of said derivatives, if any; and (d) comparing said measured derivative to a known value, whereby the ischemic event may be detected or measured. It is preferred that the solid support of step (a) be a diacetate or a phosphonate matrix. It is also preferred that the metal ion used in step (a) be nickel ion. It is further preferred that copper ion not be used in this method as it is likely to demonstrate non-specific binding to albumin thiol groups (located outside the N-terminus), possibly generating false negative results.

Metal affinity chromatography methods useful in this embodiment are within the skill in the art. For example, resins for separating proteins (including albumin) using metal affinity chromatography are described in U.S. Pat. Nos. 4,569,794; 5,169,936; and 5,656,729.

In another aspect, the derivative embodiment involves a method comprising: (a) contacting a patient sample comprising naturally-occurring albumin and optionally

albumin N-terminal derivatives with an excess of a metal salt, whereby a metal-albumin complex is formed; (b) contacting the mixture of step (a) with an antibody to said complex, said antibody being bound to a solid support; (c) separating the complex from said N-terminal derivatives, if any; (d) measuring the amount of at least one N-terminal derivative, if any; and (e) comparing the measured N-terminal derivative to a known value, whereby an ischemic event may be detected or measured. In this aspect, it is preferred that the metal ion be cobalt ion.

The step of measuring the albumin N-terminal derivatives can be carried out using antibodies (monoclonal or polyclonal) to the derivatives. The antibodies can be directed to one or more of the N-terminal epitopes for each derivative. Thus, one or more antibodies directed to one or more N-terminal epitopes may be used to measure the derivatives. Additionally, measuring can be accomplished by employing an antibody(ies) to albumin non-N-terminal epitopes. Because the partitioning step has removed all naturally-occurring albumin, any remaining albumin will be an N-terminal derivative. Antibodies used in the measuring step are labeled, preferably with an enzyme or a fluorescent label or by other methods known in the art.

The derivative embodiment methods can be carried out using kits having components adapted to provide the reactants or reagents and carry out the process steps. Where the derivative embodiment method involves excess metal ion bound to a solid support, the kit illustrated in Figure 1 can be employed. Referring to Figure 1, the diagnostic kit 20 is constructed of an upper plate 1 and lower plate 3. The lower plate 3 has 1-2 elongated solid supports 6 (e.g., nitrocellulose) with a sample application filter 4 upon which a patient sample is applied through sample port 2. The filter 4 and port 2 may be positioned such that the filter 4 is common or shared by both elongated solid supports 6. The filter 4 removes cells (red and white blood cells, platelets, etc.), permitting plasma to flow through to supports 6. The patient sample migrates from the filter at the first end of each of the elongated solid supports 6 to the second ends at the end of process indicators 18. The first solid support 6 provides a test function and the second provides a control function. The solid support providing a test function has an area 8 of immobilized metal ion to which naturally-occurring

albumin binds. The albumin N-terminal derivatives continue to migrate down the solid support 6 to an area 10 containing ligand. In preferred embodiments, the ligands at area 10 are antibodies to albumin N-terminal derivatives and/or antibodies to naturally-occurring albumin. An antibody to naturally-occurring albumin may be used at area 10 provided it is directed to an epitope that is not located at the N-terminus of naturally-occurring albumin, so that it may bind to the derivatives. An antibody at area 10 to an albumin N-terminal derivative refers to an antibody directed to an N-terminal epitope of the derivative, such that the antibody is specific (i.e., recognizes only) the particular albumin N-terminal derivative. An advantage of including antibodies to albumin N-terminal derivatives at area 10 is that the amount of each or all N-terminal derivatives can be measured. Measurement of each derivative may permit a more accurate assessment of the degree and timing of the ischemic event. For example, a relatively higher concentration of the derivative lacking four N-terminal amino acids may reflect a greater degree or a longer duration of ischemia than a second sample where another derivative (e.g., albumin lacking only its N-terminal Asp residue) is more prevalent. Although the relative order of appearance of each derivative during the course of an ischemic event has not yet been determined, it will be possible to do so upon correlation of derivatives observed in patient samples with clinical observations of patients from whom the samples have been derived.

In the control (second) elongated solid support 6, an area 11 containing ligand to albumin is provided to detect all albumin, naturally-occurring or N-terminal derivatives, in the sample. Thus, the antibody at area 11 is directed to an albumin epitope that is not located at the N-terminus of albumin. The antibody or antibody mixture at areas 10 and 11 should be the same for control purposes.

The test and control results can be observed through ports 12 and 14, respectively. The binding of albumin or albumin N-terminal derivatives to antibody is detected by methods known in the art such as sandwich assays, enzyme assays or color indicators. For example, a labeled antibody may be added through ports 12 and 14 to bind to any albumin that is bound to antibody attached to areas 10 and 11. The label on the added antibody may be, for example, alkaline phosphatase, a commonly

used reporter enzyme which reacts with synthetic substrates such as 1,2-doxetane or p-nitrophenylphosphate to yield detectable products. Alternatively, a protein coloring reagent such as bromo cresol purple or bromo cresol green may be present in areas 10 and 11 or added through ports 12 and 14.

Finally, an end of process indicator 18 at the second end of each elongated solid support 6 may be employed to assure completion of the test, i.e., that a sufficient volume of biological sample has passed down each elongated solid support 6 for the test to be completed. Suitable end of process indicators 18 include pH indicators and conductance indicators as is known in the art.

The kit illustrated in Figure 1 can also be used where the derivative embodiment method employs a solid-support bound antibody to the albumin-metal complex. Referring again to Figure 1, the patient sample is first mixed with excess metal ion aqueous solution, whereby naturally-occurring albumin-metal complexes are formed, and then applied to the filter 4 at the first end of the elongated solid supports 6. As the sample migrates down the test (first) elongated solid support 6, it encounters area 8 between the first and second ends which has immobilized antibody to the albumin-metal complex. The albumin-metal complex binds to area 8, and the N-terminal derivatives continue migration to area 10 containing ligand to albumin which is proximate the second end. The ligand at area 10 can be an antibody directed to an albumin epitope that is not located at the naturally-occurring N-terminus, or can be antibodies to derivative N-terminal epitopes. An end of process indicator 18 can also be present at the second end of the first elongated solid support. A second or control elongated solid support 6 can also be present in the kit 20 with an area 11 having immobilized antibody to the albumin located between the first and second ends.

The subject invention provides additional kit embodiments suitable for the derivative embodiment method employing the solid support bound antibody to albumin-metal complex. Referring now to Figure 2, a kit 40 is provided containing a solid support disk or circle 28 having a centrally located sample application filter 30 for application of a patient sample that has been mixed with excess metal ion,

whereby naturally-occurring albumin-metal complexes have been formed. The circular filter is surrounded by an inner concentric ring divided into a test half 32 which contains ligand (e.g., monoclonal antibody) to albumin-metal complexes, and a control half 34 which contain no ligand. Beyond the inner concentric ring is an outer concentric ring divided into a test half 38 and a control half 36, both of which contain ligand to albumin. In area 36, ligand is provided that detects all albumin, naturally-occurring or N-terminal derivatives, in the sample. Thus, the antibody at area 36 is directed to an albumin epitope that is not located at the N-terminus of naturally-occurring albumin. In area 38, ligand to naturally-occurring albumin and/or to albumin N-terminal derivatives is likewise provided. Again, for control purposes, the antibody or antibody mixture in areas 36 and 38 should be the same.

As the patient sample radiates from the filter 30, the albumin-metal complexes bind to antibody to complexes in area 32. Filtrate from area 32 passes into area 38, where albumin N-terminal derivatives bind to antibody. Likewise, as patient sample radiates through area 34 of the control half and into area 38, all albumin present (naturally-occurring and derivative) binds to antibody present in area 36. The amount of albumin or albumin derivatives bound in area 38 is compared to a known value to determine whether an ischemic event has occurred. The amount of albumin or derivatives in area 38 can also be compared to a scale of known values, such as a color scale, to determine the degree of the ischemic event. The amount of albumin or derivatives bound in area 38 is determined by methods known in the art including sandwich assays, enzyme assays or protein color reagents.

As can be appreciated by those skilled in the art, the embodiment in Figure 2 can also be readily adapted to the derivative embodiment method in which metal ion is bound to the solid support. Specifically, the solid support area 32 would have metal ion bound thereto rather than antibody to albumin-metal complex.

Figure 3 illustrates another kit 60 suitable for the derivative embodiment method employing the solid support bound antibody to albumin-metal complex. The kit 60 comprises a circular solid support 56 with a centrally located sample application filter 50. The filter 50 is surrounded by a concentric ring which is divided

into two semi-circles. The control semi-circle contains an area 54 containing ligand to naturally-occurring albumin and albumin derivatives, preferably an antibody directed to an albumin epitope not located at the N-terminus of naturally-occurring albumin. The test semi-circle contains an area 52 containing ligand to albumin-metal complex. Thus, after a patient sample is mixed with an excess metal ion solution, whereby albumin-metal complexes are formed, it is applied to filter 50 from which it radiates to area 52, where the albumin-metal complexes bind to the ligand. In the control semi-circle, the patient sample radiates and the naturally-occurring albumin (complexes) and derivatives bind to the ligand in area 54. The ligand in area 54 is preferably a monoclonal or polyclonal antibody directed to a non-N-terminal epitope of naturally-occurring albumin. By comparing the amount of total albumin and derivatives bound to area 54 to the amount of albumin-metal complexes bound to area 52, the amount of albumin derivatives can be calculated or estimated, and an ischemic event detected or measured. The albumin or derivatives bound to antibodies on each area (52 or 54) can be detected or measured by methods known in the art including sandwich assays, enzyme assays and protein color assays.

Figure 3 can likewise be adapted to be useful in the derivative embodiment method in which metal ion is bound to the solid support, i.e., where metal ion is immobilized in area 52.

As is discussed above, a variety of antibodies are employed in the various embodiments of the subject invention. In the excess metal, endogenous copper and derivative embodiments, antibodies to albumin-metal complexes are employed. Patient antibodies specific to the albumin-metal (cobalt and nickel) complexes (including the N-terminal epitope) have been identified in occupational studies (Nieboer et al. (1984) Br. J. Ind. Med. 41:56-63; Shirakawa et al. (1992) Clin. Exp. Allergy 22:213-218; Shirakawa et al. (1990) Thorax 45:267-271; Shirakawa et al. (1988) Clin. Allergy 18:451-460; and Dolovich et al. (1984) Br. J. Ind. Med. 41:51-55). Additionally, rabbit antibodies to human albumin-metal complexes have also been generated (Veien et al. (1979) Contact Dermatitis 5:378-382). Therefore,

antibodies to albumin-metal complexes for use in the subject methods either already exist in the art or would be readily obtainable using known methods.

In addition to the foregoing antibodies, the derivative embodiment may also use antibodies to one or more of the albumin N-terminal derivatives. As is set forth in the Examples, it has been found that the albumin derivatives that lack four, three, two and even one N-terminal amino acid have lost the capacity to bind to cobalt. Additionally, full-length albumin that has been acetylated at its Asp residue also cannot bind to cobalt. As is appreciated by the skilled artisan, antibodies that are specific to (i.e., recognize only) each of these derivatives can be obtained using known monoclonal antibody technology. Adjuvants such as KLH may be used to enhance immunogenicity.

Applications, embodiments and methods of the present invention comprising one or more of the aforementioned methods of the present invention include: A method for ruling-out the existence of ischemia in a patient, comprising application of any of the aforementioned methods, including application of any of the subject methods wherein said patient possesses one or more cardiac risk factors, said cardiac risk factors being selected from the group consisting of: age greater than 50, history of smoking, diabetes mellitus, obesity, high blood pressure, high cholesterol, and strong family history of cardiac disease. A variant thereof, comprises subjecting the patient to an exercise treadmill test followed by a second application of the same method, followed by a comparison of the results of the two applications. Comparison of the before and after ischemia diagnostic tests will reveal whether the ischemic event is induced only under the elevated metabolic conditions of exercise. This method may be used to detect the existence of ischemia provoked by exercise in an otherwise asymptomatic patient.

Other embodiments, applications and variants of the present invention include a method for ruling-out the occurrence of an temporally-limited ischemic event in a patient comprising application of any of the subject diagnostic methods; a method of detecting the existence of ischemia in an asymptomatic patient comprising application of any of the subject diagnostic methods; a method for the evaluation of patients

suffering from stroke-like signs to determine the occurrence or non-occurrence of a stroke, comprising application of any of the subject diagnostic methods; a method for distinguishing between the occurrence of an ischemic stroke and a hemorrhagic stroke, comprising application of any of the subject diagnostic methods; and a method for assessing the efficacy of an angioplasty procedure, comprising application of any of the subject diagnostic methods.

The present invention also provides a method for evaluation of a patient presenting with angina or angina-like symptoms to detect the occurrence or non-occurrence of a myocardial infarction, comprising application of any of the subject diagnostic methods and application of an electrocardiographic test, followed by correlation of the results of the application of the diagnostic method with the results of the electrocardiographic test to determine the occurrence or non-occurrence of a myocardial infarction. Preferred electrocardiographic tests are E.C.G., E.K.G. and S.A.E.C.G. tests.

Another method of the present invention is a method for supplementing electrocardiographic results to determine the occurrence or non-occurrence of an ischemic event, comprising application of any of the subject diagnostic methods and application of an electrocardiographic test, followed by correlation of the results of application of the diagnostic method with the results of said electrocardiographic test to determine the occurrence or non-occurrence of an ischemic event. A variant thereof, comprises application of the method wherein said patient is undergoing surgery.

A further method of the present invention is a method for comparing levels of ischemia in patients at rest and during exercise is also taught by the present invention, comprising application of the following steps at designated times: (a) application of any of the subject diagnostic methods at a first designated time, (b) administration of an exercise treadmill test followed by a second application of the same diagnostic method employed in step (a), (c) comparing the results of the application of the diagnostic method prior to administration of the exercise treadmill test with the results of the application of the diagnostic method after administration of the exercise

treadmill test, and (d) repeating steps (a) through (c) at additional designated times wherein, results obtained at designated time are compared. This embodiment may be used to evaluate patients with known or suspected ischemic conditions, to assess the patency of an in-situ coronary stent and to assess the efficacy of an angioplasty procedure. Preferred designated time intervals are three months, six months or one year.

The present invention also teaches a method for assessing the efficacy of thrombolytic drug therapy, comprising the application of any of the subject diagnostic methods; and a method for detecting in a pregnant woman the occurrence of placental insufficiency, comprising application of any of the subject diagnostic methods.

The subject invention also includes calibration standards which have known molar ratios of albumin and metal and are useful in calibrating analyzers or kits that employ the subject methods. In one embodiment, the calibrator compositions are standards to be used to generate standard curves for calibration of clinical chemistry analyzers such as the Beckman CX-5™, Roche Cobas Mira™ and Dimension XL™. These analyzers can each detect or measure ischemic events based on the colorimetric version of the excess metal embodiment described herein. The calibrator compositions can also be used to calibrate analyzers such as atomic absorbance or atomic emission spectrophotometers. The calibrator compositions have preselected or predetermined ratios of naturally-occurring albumin and metal ion. In preferred embodiments, the albumin is human, the solution is buffered (e.g., Tris or HEPES), the pH is about 7-8, and the metal is divalent and is selected from the group consisting of cobalt, nickel and copper. Aliquots of these calibrators, under specific conditions, produce a defined absorbance at 470-500 nm, i.e., a standard curve.

The albumin that is used in the calibrators is substantially all naturally-occurring. By "substantially all," it is meant that at least 70%, and with increasing preference, at least 80%, 90% and 95% by weight, of the albumin is naturally-occurring. Without wishing to be bound by theory, it is believed that when the calibrator compositions are placed in solution, the metal ion becomes primarily bound

to the N-terminus of the albumin, although it is possible that a minor amount of metal ion can be bound to thiol or other groups located on the albumin.

The calibrators are typically manufactured by starting with initial concentrated solutions of albumin and metal salt, and then mixing these concentrates in defined ratios to obtain desired molar ratios of albumin and metal concentrations in the resulting calibrator solutions.

To generate the standard curve for the colorimetry-type analyzers, each of the calibrator solutions is mixed with a known, constant amount of excess metal salt and excess coloring reagent as described herein. Thereafter, absorbance is measured at 500 nm and blocked albumin is plotted against absorbance. Because the amount of metal originally present in the calibrator solution and the excess metal salt added are both known, the absorbance, which is associated with the excess metal ion that did not bind to albumin, can be correlated with degree of N-terminal blockage of albumin originally present in the calibrator solution. As the degree of N-terminal blockage, i.e., percentage of original metal concentration, in the calibrator solution increases, the absorbance due to excess metal ion that does not bind to albumin also increases. The relationship is linear.

To generate the standard curve for the atomic absorbance or atomic emission spectrophotometer, the calibrator solutions are applied to the analyzer. The absorbance is plotted against the original metal concentration present in each calibrator to generate the standard curve.

Thus, the calibrator solutions are designed and intended to mimic ischemic patient samples in reflecting a range of albumin that is already bound to metal ion and is unavailable for binding to exogenously added metal ion. For example, a calibrator solution that has 75% of its albumin blocked with Cu at its N-terminus has only 25% of its albumin available for binding to exogenous, excess Co. After addition of coloring reagent to react with unreacted Co, absorbance at 500 nm will be much greater than that which would be observed for a calibrator solution that is only 25% blocked with Cu at its N-terminus.

For quality control purposes, the characteristics of the calibrators can be verified by:

1. measuring their metal to albumin ratio; metal can be measured by atomic absorption, and albumin can be measured by bromo cresol green (BCG) assay;

2. using radioactive Co^{57} albumin binding assay employing a Sepharose column;

3. measuring the absorbance of the calibrators at the appropriate wavelength over time; and

4. measuring the absorbance of mixtures of calibrator solutions and excess cobalt plus coloring reagent, such as dithiothreitol (DTT).

A greater understanding of the present invention and of its many advantages may be had from the following examples, given by way of illustration. The following examples are illustrative of some of the methods, applications, embodiments and variants of the present invention. They are, of course, not to be considered in any way limitative of the invention. Numerous changes and modification can be made with respect to the invention.

EXAMPLE 1

Sample Handling Procedures for Ischemia Testing

The samples which were used in the present invention were obtained from a variety of tissues or fluid samples taken from a patient, or from commercial vendor sources. Appropriate fluid samples included whole blood, venous blood, arterial blood, blood serum, plasma, as well as other body fluids such as amniotic fluid, lymph, cerebrospinal fluid, saliva, etc. The samples were obtained by well known conventional biopsy and fluid sampling techniques. Preferred samples were blood plasma and serum and purified albumin. Purified albumin was isolated from the serum by any of the known techniques, including electrophoresis, ion exchange, affinity chromatography, gel filtration, etc.

Blood samples were taken using Universal Precautions. Peripheral venipuncture was performed with the tourniquet on less than 30 seconds (contralateral

arm from any IV fluids). Blood is drawn directly into two 10 cc Becton Dickinson Vacutainer® Sodium-Heparinized tubes and was gently inverted once to mix. If an IV port was in use, the blood was collected (after a discard sample was drawn equivalent to the dead space of usually 5 cc) into a plain syringe and dripped gently down the side of two 10 cc Becton Dickinson Vacutainer® brand tubes and gently inverted once to mix. Blood was also collected directly from the Vacutainer® tubes with special administration sets with a reservoir system that does not require a discard sample. These systems allow a draw to be taken proximal to the reservoir.

Plasma tubes were centrifuged within 2 hours of the draw. (Note, collected serum was clotted between 30-120 minutes at room temperature (RT) before centrifugation. The inside of the serum tube was ringed with a wooden applicator to release the clot from the glass before centrifugation. If the subject was taking anti-coagulants or had a blood clotting dysfunction, the sample was allowed to clot longer than 60 minutes, between 90-120 minutes was best.) The tubes were centrifuged for 10 minutes at RT at 1100g (<1300g). Collected samples were pooled in a plastic conical tube and inverted once to mix.

If the sample was not used within 4 hours of centrifugation, the sample was frozen. Alternatively, separated serum was refrigerated at 4° C until tested, but was tested within 8 hours (storage over 24 hours may have resulted in degradation of the sample). "Stat" results (obtained within 1 hour of completion of centrifugation step) were preferred. The following percent differences for the ischemia test were measured using plasma and serum samples ≤ 8 hours and ≤ 24 hours after collection. Delayed test results were compared to stat test results on the same patient sample and the mean percent differences (and standard deviations) were as given below:

Storage and Delayed Testing Data for the Ischemia Test

		≤ 8 hrs. vs. stat		≤ 24 hr. vs. stat*	
	Plasma	n	20	n	23
	(stored at	% diff	-5.3%	% diff	-4.8%
	room temp)	S.D.	.094	S.D.	.090
	Plasma	n	18	n	40
	(stored at	% diff	1.7%	% diff	1.0%
	4° C)	S.D.	.070	S.D.	.094
	Serum	n	16		
	(stored at	% diff	-12.8%	(not enough	
	room temp)	S.D.	.157	samples)	
	Serum	n	14	n	24
	(stored at	% diff	-7.3%	% diff	-2.7%
	4° C)	S.D.	.040	S.D.	.210

* ≤ 24 hr. test results given here are a total that include the ≤ 8 hr. test sample results.

EXAMPLE 2

Test Method for Detecting Occurrence of Ischemic Event Using Cobalt Binding

The ischemia test (cobalt version) was run as follows: 200 µl of patient sera was added to each of two tubes each containing 50 µl 0.1% $CoCl_2 \bullet 6H_2O$. The mixture was allowed to react at room temperature (18-25° C), or higher, for 5 or more minutes. Thereafter 50 µl 0.01 M dithiothreitol (DTT) was added to one of the two tubes (the "test tube") and 50 µl 0.9% NaCl was added to the second tube (the "background tube"). After two minutes, 1 ml 0.9% NaCl was added to both tubes. A470 spectroscopy measurements were taken of the two tubes. The ischemia test was considered positive if the optical density was greater than or equal to .400 OD (or alternatively a clinically derived cut-off) using a spectrophotometer at OD 470nm.

Equivalent materials which may be used as alternatives include any of the transition metals. Ferrozine or other compounds with an affinity to cobalt can be substituted for DTT and/or any cobalt or metal coloring reagent. $CoCl_2 \bullet 6H_2O$, for instance, can be utilized. The optimal range for cobalt binding to albumin is from pH 7 to pH 9, with a range of pH 7.4-8.9 being most preferred; pH 9 is optimal for cobalt interaction with the color reagent. The amount of serum sample can also vary, as can the amounts of $CoCl_2 \bullet 6H_2O$ and DTT and ferrozine. Critical, however, is that the amount of cobalt used be in excess of the amount of albumin and that the DTT or ferrozine be in excess of the cobalt.

EXAMPLE 3

Test Method For Detecting Occurrence of Ischemic Event Using Measurement of Copper

Albumin was purified from .2 cc of human serum or plasma using an ion exchange method to produce approximately 8 mg of purified albumin. A buffer having a pH in the range of 7 to 9 was added. The amount of copper present in the sample was then measured by direct spectrophotometric and potentiometric methods, or by any of several other known methods, including atomic absorption, infrared spectroscopy, HPLC and other standard or non-standard methods, including radioactive tracer techniques. The proportion of copper to albumin can be then used as a measure of ischemia, the greater the proportion, the higher the ischemia value.

EXAMPLE 4

Test Method for Ruling-out The Existence of Ischemia in a Patient

The following protocol is designed to rule out ischemic conditions in healthy appearing patients who describe prior symptoms of occasional chest pain or shortness of breath.

First, a medical history (including a detailed history of the present and past medical problems and risk factors for ischemic heart disease), physical exam, and vital signs are obtained. If the patient has any cardiac risk factor for ischemic heart disease (age > 50, smoking, diabetes mellitus, obesity, high blood pressure, elevated

low density lipoproteins, high cholesterol, and strong family history of cardiac disease), the physician is instructed to order a resting twelve-lead EKG and a chest x-ray. If the twelve-lead EKG shows evidence of an acute myocardial infarction (AMI), the patient is immediately transported to a hospital for intensive cardiac treatment. If the twelve-lead EKG does not show evidence of (AMI), the patient will be scheduled for an outpatient twelve-lead EKG exercise treadmill within the next few days. A blood sample should be drawn immediately before and again after the exercise treadmill test and the ischemia test run on each sample.

If the exercise treadmill test shows definite evidence of cardiac ischemia, usually seen by characteristic changes of the ST segments, dramatic abnormalities of pulse or blood pressure, or anginal chest pain, the patient should be treated for cardiac ischemia and referred to a cardiologist for possible coronary angiogram and angioplasty. If the exercise treadmill test does not show any evidence of cardiac ischemia, or the findings are equivocal, but the ischemia test is abnormal, the patient similarly should be treated for cardiac ischemia and referred to a cardiologist for possible coronary angiogram and angioplasty. (Absent the present invention, such patients with moderate to high cardiac risk factors would be referred to a cardiologist for further (typically invasive) cardiac testing).

If the exercise treadmill test does not show any evidence of ischemic heart disease, or the findings are equivocal, and the ischemia test is normal, the patient may be sent home with no evidence of cardiac ischemia. In comparison, prior to the present invention, in the case where the exercise treadmill test does not show any evidence of cardiac ischemia, or the findings are equivocal, patients with low risk for cardiac ischemia typically would not have any other tests ordered. In such cases, the physician is taking a calculated risk. It is well documented in the medical literature that at least 25 to 55 percent of patients (higher in females) will have some ischemic heart disease which is not found with routine exercise treadmill testing.

EXAMPLE 5

Test Method for Evaluating Patients with Angina to Rule-out the Occurrence of An Ischemic Event

In this study, clinical criteria (EKG changes, elevated cardiac enzymes or markers, positive thallium treadmill or positive angiogram) were used to determine the presence or absence of ischemia in patients presenting with chest pain. Ischemic patients were those with at least one clinical finding positive for ischemia. Normal patients were those for whom clinical findings were negative, as well as normal volunteers with no history or symptoms of cardiac or cerebral ischemia.

Blood samples were taken from 139 subjects who either presented to emergency departments of several hospitals with chest pain or normal volunteers. Blood was drawn into plain red top tubes and, after ten minutes, the clotted blood was centrifuged to separate the serum. Serum was refrigerated at 4° C until tested. If the sample would not be used within 4 hours of centrifugation, it was frozen, but in no case was testing delayed more than 3 days.

Samples were centrifuged for 5-10 minutes in an analytical centrifuge immediately before testing. 200 µl off each sample was aliquoted in triplicate with an additional tube to be used as a Blank (no DTT) control into borosilicate glass tubes. Also aliquoted was 200 µl of a Standard, such as Accutrol or HSA, in triplicate plus a Blank control. At 10 second intervals, 50.0 µl of 0.10% $CoCl_2$ (store working stock and stock at 4° C) was added to each tube. Solution was added to the sample, not glass, and tubes were "flicked" to mix.

After 10.0 minutes (starting with the first tube to which cobalt solution was added) an additional 50.0 µl of 0.9% NaCl was added to the two Blank tubes using the appropriate 10 second intervals. 50.0 µl of 0.01 M DTT was additionally added to the Plasma (not Blank) tubes in their appropriate 10 second intervals. Of note, it is preferred that DTT be made fresh weekly (6 mg per 4 ml H_20) and stored at 4° C.

After 2 minutes (starting with the first tube to which cobalt solution was added) 1.0 ml of 0.9% NaCl solution was added to each tube, using the appropriate 10 second intervals. Tubes were agitated to mix. In the event that there were too many

tubes to finish the test tubes in 10 second intervals, reagents were added to the "Blank" tubes without timing.

The optical density of each sample set was read using the set's Blank to read absorbance at 470 nm. The cuvette was checked for air bubbles before reading and washed with H_20 between sets. The ischemia test was considered positive if the optical density was greater than or equal to .400 using the spectrophotometer at OD 470 nm.

The results of the ischemia test compared to the diagnosis determined by clinical criteria are as described in the chart below. Four false negatives and three false positives were reported.

Clinical Diagnosis		Ischemia Test	
		+	—
+	99	95	4
—	40	3	37

Study results demonstrated that the ischemia test marker has a higher value in patients with clinically diagnosed ischemia. The diagnostic accuracy of the ischemia test for the chest pain study was above 90 percent (sensitivity, 96.0%; specificity, 92.5%; predictive value, (+)96.9%; predictive value, (-) 90.2%).

EXAMPLE 6

Test Method For Evaluation of Patients Suffering From Chest Pain to Determine the Occurrence or Non-occurrence of a Myocardial Infarction

The following study is proposed to test the ability of the present invention to detect ischemia in the initial hours following the onset of chest discomfort suspicious for cardiac ischemia. The cobalt version of the test is used.

The patient population is limited to male or female persons, 30 years or older, who present to the Emergency Department with complaints of chest discomfort of less than four hours in duration for reasons independent of the study. Patients will be

excluded from the study if they meet any of the following criteria: (1) known concurrent non-cardiac ischemic disease(s), including but not limited to transient ischemic attacks, cerebral vascular accident, peripheral vascular disease, intermittent claudication, bowel ischemia, and severe renal failure; (2) definite radiological evidence of a cause of chest discomfort that is other than cardiac ischemia, such as, but not limited to, pneumonia, pneumothorax, and pulmonary embolus; or (3) chest discomfort temporally related to local trauma.

All standard evaluation and treatment appropriate for emergency department patients with suspected cardiac ischemia will be followed at all times. The drawing of blood for the study will not in any manner modify the standard treatment protocol. Within these parameters, a pre-treatment evaluation will be conducted, which will include documentation of all current medications, documentation of previous medical history, EKG, laboratory and radiographic test results, and documentation of most recent vital signs and a physical examination.

The study consists of drawing an extra blood sample at the time of admission to the emergency department. Samples are collected from a catheter that is already in place for intravenous access or alternatively by venipuncture. Collection and administration of the ischemia test is as described in Example 5 herein.

EXAMPLE 7

Test Method For Detection of Ischemia in Patient at Rest and During Exercise

The primary objective of this trial was to employ and test the sensitivity of the ischemia test at various time points, before, during and after an exercise thallium treadmill test. Preliminary data has shown that the blood level of the ischemia test (i.e., absorbance, cobalt excess metal embodiment) rises immediately after an ischemic event. The purpose of this pilot investigation is to determine the magnitude of this rise in level of the ischemia test during a test to define the presence or absence of a cardiac ischemic event, said test being the exercise thallium treadmill test. While it is possible that patients scheduled for exercise thallium treadmill test may have already experienced an ischemic event, preliminary data indicates that a further,

significant decline in cobalt binding (and an increase in the serum absorbance or unbound metal ion) will occur if tissue ischemia is induced during the exercise thallium treadmill test.

Patients already scheduled for an exercise thallium treadmill test were asked to give their consent for participation which required two tubes of blood (20 cc's) to be drawn up to 5 (five) times before, during and after the exercise thallium treadmill test. Eligible patients consisted of patients who met all of the following criteria: (1) Age: 18 years or older; (2) Male or female; (3) able to provide written informed consent; and (4) referred for exercise thallium treadmill test for reasons independent of this investigation. Patients were excluded from participation in the study if they met any of the following criteria: (1) known concurrent non-cardiac ischemic disease including, but not limited to: transient ischemic attacks, cerebral vascular accident, acute myocardial infarction and intermittent claudication; (2) inability to complete the standard protocol for the exercise portion of the exercise thallium treadmill test; or (3) cardiac arrest during the exercise portion of the exercise thallium treadmill test.

Prior to administration of the exercise thallium treadmill test, a pretreatment evaluation was conducted which included documentation of all current medications, documentation of previous medical history, EKG, laboratory and radiographic test results, and documentation of most recent vital signs and physical examination.

The standard exercise thallium treadmill test procedure was followed at all times. In no instance was the drawing of the additional blood samples for the purpose of the study permitted to subject the patient to additional risk (beyond the drawing of blood), or to in any manner modify the treatment of the patient.

The "standard" exercise thallium treadmill test procedure comprised generally the following: The patient was brought to the exercise test room in a recently fasting state. After initial vital signs and recent history was recorded, the patient was connected to a twelve-lead EKG monitor, an intravenous line was established and the patient was instructed in the use of a treadmill. With the cardiologist in attendance, the patient walked on the treadmill according to the standard Bruce protocol: starting at a slow pace (approx. 1.7 mph) and gradually increasing both the percent grade

(slope) of the treadmill and the walking speed at three minute intervals up to a maximum of 5.5 mph at 20° grade. Termination of the exercise portion on the exercise thallium treadmill test occurred at the discretion of the cardiologist based on patient symptoms, EKG abnormalities, or the attainment of ò 85% maximal heart rate.

With the patient near maximal effort on the treadmill, approximately 3 mCi of thallium^{201} was injected intravenously while the patient continued to exercise for approximately one more minute. At the end of exercise, single photon emission computerized tomography (SPECT) was used to scan the patient's myocardium for any perfusion defects. Following recovery, between 2 and 4 hours after exercise, a smaller amount of thallium^{201} (approximately 1.5 mCi) was re-injected for repeat SPECT scan. EKG's and SPECT scans were analyzed for ischemic criteria. The SPECT scans may show fixed and reversible perfusion defects. The reversible perfusion defects indicate ischemia and the fixed defects indicate myocardial scarring.

The study consisted of drawing blood samples on 3 occasions during the exercise thallium treadmill procedure. Two tubes of blood (approximately 4 teaspoons) were collected before the exercise test, immediately after exercise, and between 1 and 4 hours after exercise. Blood samples were collected from the catheter already in place for the exercise thallium treadmill procedure or alternatively by venipuncture. Note: Radiation Protection /Safety Considerations -- Blood drawn following thallium^{201} injection was routinely considered safe because the amount injected was approximately 3 mCi and, for all practical purposes, the dilution into the systemic circulation reduces the sample level to less than 0.67 nanoCi per cc.

Standard patient follow-up was conducted according to clinical practice. Patients who had subsequent coronary angiograms after being enrolled in this exercise thallium treadmill test study had all resultant coronary angiogram information obtained recorded to verify the exercise thallium treadmill test results.

All clinical and research laboratory testing procedures were performed in a blinded fashion.

Of the 59 patients enrolled (plasma and serum samples tested by the ischemia test method), 11 patients were deleted because of one of the following reasons: a chronically occluded coronary artery and no sample collected later than one hour after exercise, a clinical history of exercise leg pain (claudication), hemolyzed baseline blood samples, patient did not continue with the exercise study or did not agree to further blood tests, patient received an exercycle thallium test instead of a treadmill thallium test and one patient whose chest pain was later determined to be due to pneumonia.

Of the remaining 48 patients, 23 had no history of known ischemic heart disease, 23 had prior ischemic heart disease requiring angioplasty or coronary artery bypass grafts and 2 had prior myocardial infarctions but did not receive angioplasty or coronary artery bypass grafts. In the subgroup of 23 patients with no prior history of ischemic heart disease (using a total outcome score of ≥ 9 and a $\geq 4.7\%$ increase in Ischemia Test values (i.e., absorbance associated with unbound excess metal ion) either one or three hours after exercise as positive for ischemia) there were 2 true positives, 15 true negatives, 6 false positives and 0 false negatives for a sensitivity of 100% and a specificity of 72%.

Using the same criteria for positive exercise thallium treadmill and Ischemia Test results, the entire 48 patients (including patients with and without a prior history of ischemic heart disease) had 6 true positives, 29 true negatives, 11 false positives and 2 false negatives for a sensitivity of 75% and a specificity of 73%.

Changing the positive criteria to a total thallium treadmill outcome score of ≥ 10 and a $\geq 5.4\%$ increase in Ischemia Test values one hour after exercise for the entire 48 patients (including patients with and without a prior history of ischemic heart disease) gave 3 true positives, 37 true negatives, 7 false positives and 1 false negative for a sensitivity of 75% and a specificity of 88%.

EXAMPLE 8

Assessing Efficacy of an Angioplasty Procedure

Percutaneous transluminal coronary angioplasty ("PTCA"), also referred to as coronary artery balloon dilation or balloon angioplasty, is an established and effective therapy for some patients with coronary artery disease. PTCA is an invasive procedure in which a coronary artery is totally occluded for several minutes by inflation of a balloon. The inflated balloon creates transient but significant ischemia in the coronary artery distal to the balloon. The result, however, is a widening of a narrowed artery.

PTCA is regarded as a less traumatic and less expensive alternative to bypass surgery for some patients with coronary artery disease. However, in 25 to 30 percent of patients, the dilated segment of the artery renarrows within six months after the procedure. In these cases, either repeat PTCA or coronary artery bypass surgery is required. Additionally, complications from angioplasty occur in a small percentage of patients. Approximately, 1 to 3 percent of PTCA patients require emergency coronary bypass surgery following a complicated angioplasty procedure.

The present invention addresses both problems by providing a means for monitoring on-going angioplasty procedures and by providing a mechanism for monitoring the post-angioplasty status of patients.

Twenty-eight patients already scheduled for emergent or elective angioplasty had blood samples (20 ml) drawn just prior to undergoing PTCA ("baseline") at 6, 12 and 24 hours after the last balloon deflation, and three tubes (25ml) at 1 minute and 6 minutes after the last balloon deflation. Collection and administration of the test was as described in Example 5 herein. A detailed description of the angioplasty procedure was also recorded so the magnitude of 'downstream' ischemia could be estimated. This included catheter size, number of inflations, inflation pressure, duration of inflation, number of vessels involved and location.

The eligible patient population consisted of male or female patients who met all of the following criteria: (1) 18 years or older; (2) referred for PTCA for reasons independent of the study; (3) able to give written, informed consent; and (4) and did

not possess any of the exclusionary criteria. Patients were excluded if they met any of the following criteria: (1) patients who were to have PTCA performed with a perfusion catheter; (2) patients with known, concurrent ischemic disease including, but not limited to transient ischemic attacks, cerebral vascular accident, acute myocardial infarction and intermittent claudication. Prior to PTCA, a pretreatment evaluation was conducted which included documentation of all concurrent medications and the taking of a blood sample for ischemia test administration and baseline (this occurred after the patient had been heparinized and the sheath placed).

The standard PTCA protocol was followed at all times. In no instance was the drawing of the additional tubes of blood permitted to subject the patient to additional risk (beyond the drawing of the blood), or modify the standard protocol.

The "standard" PTCA protocol generally comprised the following: The patient was transported to the cardiac catheterization laboratory in the fasting state. The right groin draped and prepped in the usual sterile fashion. Local anesthesia was administered consisting of 2% lidocaine injected subcutaneously and the right femoral artery entered using an 18 gauge needle, and an 8 French arterial sheath inserted over a guide wire using the modified Seldinger technique. Heparin, 3000 units, was administered I.V. Left coronary cineangiography was performed using Judkins left 4 and right 4 catheters, and left ventricular cineangiography performed using the automated injection of 30 cc of radiocontrast material in the RAO projection. After review of the coronary angiography, PTCA was performed.

The diagnostic cardiac catheter was then removed from the femoral sheath and exchanged for a PTCA guiding catheter which was then positioned in the right or left coronary ostia. An additional bolus of intravenous heparin, 10,000 units, was administered. A coronary guidewire, usually a 0.014 inch flexible tipped wire, was then advanced across the obstruction and positioned distally in the coronary artery. Over this guidewire, the balloon inflation system was inserted, usually consisting of a "monorail" type balloon dilation catheter. Sequential balloon inflations were made, with angiographic monitoring between inflations. The duration of the inflations

varied among operators, but averaged approximately 45 - 60 seconds; occasionally prolonged inflations between 3 and 15 minutes were performed.

When it was determined that adequate opening of the coronary stenosis had been achieved, the balloon catheter was fully withdrawn and coronary angiograms performed with and without the guidewire in position. If no further intervention was believed to be necessary, the sheath was then sewn into position and the patient transported to either the intensive care unit or observation unit. The sheath was removed after approximately 6 hours and firm pressure applied with a C clamp or manual pressure. The patient remained at bed rest for approximately 6 hours after sheath removal.

Standard patient follow up was conducted according to clinical practice.

As stated, sample collection and administration of the ischemia test occurred essentially as described in Example 5 herein. The test technician was masked to the time the PTCA sample was taken.

Compared to baseline, 26 of the 28 tested patients demonstrated increased ischemia values after balloon inflation. The remaining two patients registered false negatives, both of which started with baseline values above .400. The mean increase in the ischemia test value from baseline to balloon inflation was 15.2%. Of the 21 patients that had 5 hour samples tested, all but three demonstrated a decreased ischemia test value compared to that measured during balloon inflation. Study results demonstrated that the ischemia test marker rises almost immediately following controlled onset of ischemia during the angioplasty procedure. The rapid rise of the marker during balloon inflation and its descent over a five hour period correlated with the controlled start and stop of ischemia. The diagnostic accuracy of the study was 96 percent.

EXAMPLE 9

Evaluation of Post-Myocardial Infarction Patients

In a second study, three subsets of patients -- patients without acute myocardial infarction (NonAMI), patients with acute myocardial infarction (AMI),

and patients without AMI with significant collateral circulation (NonAMI collateral) -- all of whom were undergoing emergent or elective angioplasty had blood samples collected prior to PTCA, immediately after balloon deflation, 6 hours after the procedure, and 24 hours after the procedure. A total of 63 patients were tested. The standard PTCA protocol (as described in Example 8) was followed.

During PTCA, blood was drawn into a syringe and then transferred to sodium-heparinized tubes. Post PTCA samples were drawn into green top sodium-heparinized tubes. In all other regards, sample collection and administration of the ischemia test occurred essentially as described in Example 5 herein. The test technician was masked to the time the PTCA sample was taken.

The ischemia test was considered positive if it increased between baseline and immediately after balloon angioplasty. The results of the study showed a statistically significant rise (p=0.0001) in the ischemia test marker following balloon angioplasty and a return to baseline within 24 hours. The mean percent increase for all patients in the study was 9.4%. .

TIME POINT	N	MEAN	SD	MEAN DIFF FROM BASELINE	SD	MEAN % DIFF FROM BASELINE	SD	P-VALUE
Baseline	62	.354	.0424	.	.	.	.	.
Immed. post PTCA	63	.385	.0411	.0310	.0382	9.4%	.1178	.0001
6 hours post PTCA	57	.368	.0513	.0150	.0505	5.0%	.1507	.0167
24 hours post PTCA	43	.363	.0474	.0090	.0444	3.2%	.1312	.1221

% CHANGE FROM BASELINE	WITH AMI			WITHOUT AMI			T-TEST
	N	MEAN	SD	N	MEAN	SD	P
Immed Post PTCA	19	.083	.137	41	.101	.111	.0001
6 hrs Post PTCA	15	.091	.137	39	.027	.153	.2676
24 hrs Post PTCA	14	.130	.158	27	.019	.081	.2240

A side branch occlusion ("SBO") occurs when, as a result of balloon inflation, a side artery becomes obstructed, causing loss of blood flow and ischemia distal to the occlusion. Patients with side branch occlusion (SBO) were predicted to have more ischemia than those without. Patients were assigned to the SBO subset if their cardiologist indicated they had significant SBO.

Study results showed significantly higher ischemia test values immediately after and 6 hours after PTCA in patients with SBO. The following data includes patients in all study subsets. The number of patients varies because investigators were not always able to obtain blood samples at all four draw times.

% CHANGE FROM BASELINE	WITH SBO			WITHOUT SBO			T-TEST
	N	MEAN	SD	N	MEAN	SD	P
Immed Post PTCA	8	.228	.144	51	.076	.102	.0005
6 hrs Post PTCA	8	.150	.156	45	.033	.149	.0480
24 hrs Post PTCA	8	.168	.222	33	.013	.098	.1500

EXAMPLE 10

Assessment of the Patency of *In-situ* Coronary Stent

Coronary stents may be inserted during angioplasty and left in place on a permanent basis in order to hold open the artery and thus improve blood flow to the heart muscle and relieve angina symptoms. Stent insertion consists of the insertion of a wire mesh tube (a stent) to prop open an artery that has recently been cleared using angioplasty. The stent is collapsed to a small diameter, placed over an angioplasty balloon catheter and moved into the area of the blockage. When the balloon is

inflated, the stent expands, locks in place and forms a rigid support to hold the artery open.

Stent use has increased significantly in just the past year, and is now used in the vast majority of patients, sometimes as an alternative to coronary artery bypass surgery. A stent may be used as an alternative or in combination with angioplasty. Certain features of the artery blockage make it suitable for using a stent, such as the size of the artery and location of the blockage. It is usually reserved for lesions that do not respond to angioplasty alone due to the reclosure of the expanded artery.

In certain selected patients, stents have been shown to reduce the renarrowing that occurs in 30–40 percent of patients following balloon angioplasty or other procedures using catheters. Stents are also useful to restore normal blood flow and keep an artery open if it has been torn or injured by the balloon catheter.

However, reclosure (referred to as restenosis) is a common problem with the stent procedure. In recent years doctors have used stents covered with drugs that interfere with changes in the blood vessel that encourage reclosure. These new stents have shown some promise for improving the long-term success of this procedure. Additionally, after a stent procedure has been done, patients are often placed on one or more blood thinning agents such as aspirin, Ticlopidine and/or Coumadin in order to prevent or prolong reclosure. Whereas aspirin may be used indefinitely; the other two drugs are used only for four to six weeks.

The present invention provides a mechanism for monitoring the functioning and patency of an *in situ* stent.

Stent patency was tested in the same study and same patient group in which post-myocardial infarction patients were studied (see Example 9). The study results showed significantly lower ischemia test values immediately after and 6 hours after PTCA for those patients with stents. The following data includes patients in the NonAMI subset only. The number of patients varies because investigators were not always able to obtain blood samples at all four draw times.

% CHANGE FROM BASELINE	WITH STENT			WITHOUT STENT			T-TEST
	N	MEAN	SD	N	MEAN	SD	P
Immed Post PTCA	37	.089	.105	4	.210	.117	.0373
6 hrs Post PTCA	36	.009	.139	3	.243	.153	.0087
24 hrs Post PTCA	26	.022	.080	1	.071	NA	NA

EXAMPLE 11

Diagnosis and Assessment of Arrhythmic /Dysrhythmic Patients

The present invention provides a rapid method for assessing arrhythmias and diagnosing and measuring dysrhythmias.

Rapid assessment and treatment of arrhythmias is key to a successful outcome: if treated in time, ventricular tachycardia and ventricular fibrillation can be converted into normal rhythm by administration of an electrical shock; alternatively, rapid heart beating can be controlled with medications which identify and destroy the focus of the rhythm disturbances. If an arrhythmia is not promptly diagnosed and treated, a stroke may be the likely result. Arrhythmia prevents the heart from fully pumping blood out of the heart chambers; the undisgorged blood remaining in the heart chamber will pool and clot. If a piece of the blood clot in the atria becomes lodged in an artery in the brain, a stroke results. About 15 percent of strokes occur in people with atrial fibrillation.

Traditionally, electrocardiography, also called ECG or EKG, is used to diagnosis the occurrence of an arrhythmia. (Also utilized are the "12 lead EKG" and signal-averaged electrocardiogram (S.A.E.C.G.), the S.A.E.C.G. to identify people who have the potential to experience a dangerous ventricular arrhythmia and the "12 lead EKG" primarily in people undergoing arrhythmias.) However, all of the electrocardiographic tests yield frequent false positive and false negative results. The present invention provides a method for supplementing all of the aforementioned electrocardiographic tests in order to reduce, if not avoid entirely, the frequency of false positive and false negative diagnoses.

Other diagnostics techniques typically used are invasive and thus possess greater risk. For instance, transesophageal echocardiography (T.E.E.) is an imaging procedure, in which a tube with a transducer on the end of it is passed down a person's throat and into the esophagus; images from TEE can give very clear pictures of the heart and its structures. Cardiac catheterization is another invasive procedure which allows for measurement and viewing of the pumping ability of the heart muscle, the heart valves and the coronary arteries. The shortcoming of these procedures, however, lies in their invasive nature.

The present invention provides a non-invasive method for diagnosis and measurement of dysrhythmias which can be used in lieu of, or in supplementation of, the aforementioned invasive procedures.

Patients with dysrhythmias undergoing PTCA were predicted to have more ischemia than those without. (Dysrhythmia is cited in the medical literature as a good indicator of ischemia.) In the 63 patient study detailed in Examples 9 and 10, patients were additionally assigned to a dysrhythmia subset if their medical record showed significant dysrhythmia during PTCA. Study results showed significantly higher ischemia test values immediately after and 6 hours after PTCA in patients with significant dysrhythmias. The following data includes patients in all study subsets. The number of patients varies because investigators were not always able to obtain blood samples at all four draw times.

% CHANGE FROM BASELINE	WITH DYSRHYTHMIA			W/O DYSRHYTHMIA			T-TEST
	N	MEAN	SD	N	MEAN	SD	P
Immed Post PTCA	5	.265	.151	57	.079	.103	.0004
6 hrs Post PTCA	5	.204	.175	51	.035	.141	.0150
24 hrs Post PTCA	5	.144	.236	37	.017	.107	.3000

EXAMPLES 12-23

Use of N-terminus Peptide Probe in the Evaluation of Ischemia

Under the present invention, a four amino acid sequence found within the N-terminus sequence of albumin is the minimum sequence required for cobalt binding. This sequence has been identified as Asp-Ala-His-Lys (abbreviated "DAHK"). The binding characteristics of this tetrapeptide have been extensively studied and it has been determined that this tetrapeptide may be used to detect the presence of ischemia.

Specifically, a biological sample containing albumin is contacted with $CoCl_2 \cdot 6H_2O$. Some of this cobalt will bind to albumin. The remaining free cobalt is then reacted with a known amount of D-A-H-K·R added to the biological sample, wherein R is any chemical group or enzyme, including no group at all or a fluorescent group, capable of being detected. Because D-A-H-K·R has a great affinity to cobalt (association constant about 10^{15}) the free cobalt will attach to it. The D-A-H-K·R differs from Co-D-A-H-K·R spectroscopically. One distinction is that Co-D-A-H-K·R has an extinction coefficient that is 1.5 to 2 times the peptide alone. This phenomenon can be used to determine that the peptide has bound to the cobalt (an increase in absorption at ~ 214 nm using HPLC or other methods).

EXAMPLE 12

To a 0.2 ml sample of blood or plasma was added 50æL 0.1 % $CoCl_2$. The mixture was incubated for 5 to 10 minutes. Thereafter, 50æL of 1 mg/ml of D-A-H-K·R was added to the sample. (R was a polymer or other substance having chemical and physical characteristics that changed when the cobalt binds to the peptide - causing a small current change or any other change that was detected.) The sample was then centrifuged (Centricon 10 or 3) for 5 minutes, followed by HPLC analysis of the filtrate using a ultrahydrogel 120, 5æ column at 60° C; isocratic run, mobile phase acetonitrile: ammonium acetate buffer 3OmM pH 8.0, 2:98; at 1 ml/minute and U.V. detection at 214 nm. The peptide peak appeared at ~ 5.88 minutes.

The same procedure was run with a peptide control (no cobalt). The difference in peak size between test (with cobalt) and control (no cobalt) was proportional to the amount of free cobalt and hence ischemia.

The following preliminary experiments illustrate the properties and critical characteristics of the peptide probe.

EXAMPLE 13

Measurement of Cobalt Binding to HSA and Octapeptide using Cold Cobalt Binding Assay

OBJECTIVE: To investigate cobalt binding to the octapeptide and human serum albumin using cold cobalt binding assay.

EXPERIMENTAL: Octapeptide synthesized at the Inorganic Chemistry Department (BAM 1, Pat Ingrey, Cambridge): NH_2-Asp-Ala-His^+-Lys^+-Ser-Glu-Val-Ala-$CONH_2$

Molecular weight: 855.4 Da.

SOLUTIONS: $CoCl_2$ 0. 1 % (w/v) = 4.2 mM; HSA 3% (w/v)(in 75 mM HEPES pH 7.4) = 0.45 mM; Octapeptide 0.965 mM (in 75 mM HEPES pH 7.4); HEPES 75 mM pH 7.4; DTT 0. 15 % (w/v); NaCl 0.85 % (w/v).

METHOD: Fifty æL 0.1 % $CoCl_2$ was added to tubes each containing 200 æL of 75 mM HEPES pH 7.4 or 0.45 mM HSA in HEPES or 0.965 mM Peptide in HEPES; the tubes were allowed to stand at room temperature for 10 minutes; 50 æL DTT 0.15 % was added to one tube (test tube) and distilled H_20 to the other (control tube); the tubes were maintained for 2 minutes at room temperature; 1 ml NaCl 0.85 % was then added; the absorbance at A470 nm of the test tube versus the blank was measured.

RESULTS:

ID	A470nm		mean A470	% bound
75 mM HEPES pH 7.4	1.087	1.083	1.085	0.0
0.45 mM HSA in HEPES pH 7.4	0.668	0.643	0.656	39.5
0.965 mM Peptide in HEPES pH 7.4	0.638	0.655	0.647	40.4

CONCLUSIONS: Under the conditions used for the binding measurements, this experiment showed that: 1. Cobalt binds to the "octapeptide" (N- Asp-Ala-His$^+$-Lys$^+$-Ser-Glu-Val-Ala); 2. However the octapeptide (0.965 mM) binds cobalt with a stoichiometry of 1:2.3.

EXAMPLE 14

Mass Spectrometry of Octapeptide after the Addition of Cobalt

OBJECTIVE: To investigate whether mass spectral study would provide molecular weight information for the octapeptide and its corresponding cobalt complex.

SOLUTIONS: Ammonium acetate 20 mM-pH 7.4 (with dilute ammonia solution); $CoCl_2$20 æM (in HPLC grade H_20); Octapeptide 9.5 æM (in HPLC grade H_20).

METHOD: 20 æM $CoCl_2$ (100 æl) was added to 9.5 æM octapeptide (100 æl) and mass spectrometry carried out.

RESULTS: The main molecular ion peak was observed at 855.4 Da, with minor peaks at 877.4 and 893.4 Da probably as a result of sodium and potassium cluster ions. After the addition of cobalt, an extra molecular ion peak was observed at 912.3 Da.

CONCLUSIONS: Octapeptide showed a molecular ion at 855 Da consistent with the expected molecular weight of the peptide moiety. Octapeptide plus cobalt complex showed a molecular ion at 912 Da suggesting that at least two protons are removed during the complex formation.

EXAMPLE 15

Spectrophotometric Analysis of the Octapeptide and Octapeptide-Cobalt Complex

OBJECTIVE: It is clear from the previous mass spectrometry evidence that cobalt forms a complex with the octapeptide with a concomitant loss of two possible protons. Metal complexes in general show distinct absorption in the UV range and in many cases these complexes show either a hypochromic or a bathochromic shift in the spectra. These shifts can be correlated to provide the energy of binding. It was therefore anticipated that the octapeptide-cobalt complexation would provide such information.

METHOD: The quartz cuvette contained 800 µl octapeptide + 200 µl H_2O(control) or $CoCl_2$ (complex). Spectra were run from 180 to 800 nm on a single beam spectrophotometer.

CONCLUSIONS: Cobalt and octapeptide individually have peak absorbances at <200 and 225 nm respectively with little overlap. Following addition of a $CoCl_2$ solution to octapeptide (1.1:1) there was no significant shift in the K_{max} (220 nm). The absorption band at this region broadened indicating complex formation, but the result could not be used to determine the binding energy (constant).

EXAMPLE 16

Mass Spectrometry of Octapeptide After the Addition of Cobalt

OBJECTIVE: To investigate whether mass spectral study would provide molecular weight information for the peptide and its corresponding cobalt complex.

METHOD: 20 or 200 æM $CoCl_2$ (100 æl) was added to 22.9 æM octapeptide (100 æl) to give ratios of cobalt: octapeptide of 1 : 1.1 and 8.7 :1 respectively. Mass spectra for the two samples were carried out as per conditions detailed in the previous experiment.

RESULTS: One major molecular ion peak was observed at 855.4 Da representing the octapeptide alone. After the addition of 20 æM cobalt to the octapeptide, two peaks were observed, a major peak at 855.3 representing octapeptide

only plus a minor peak at 912.2 Da representing octapeptide-cobalt complex. Peak ratio of free octapeptide to octapeptide-cobalt complex was 1 :0.15. A similar profile was observed following the addition of 200 æM cobalt to the octapeptide. Peak ratio of free octapeptide to octapeptide-cobalt complex was 1 : 0.9.

CONCLUSIONS: On addition of cobalt (59 Da) to the octapeptide, the molecular ion peak should have occurred at 914 Da. The actual peak occurred at 912 Da, representing the loss of two protons. On addition of increasing concentrations of cobalt the peak ratio of free octapeptide to octapeptide-cobalt complex increased.

EXAMPLE 17

The Effect of Oxygen on the Binding Capacity of Octapeptide for Cobalt

OBJECTIVE: Previous experiments have highlighted the requirement of oxygen in promoting cobalt binding to HSA. It may be anticipated that similar effects could be observed in the manner of cobalt binding to the octapeptide.

METHOD: Octapeptide-cobalt complex (no oxygen): HPLC grade H_20 was bubbled with 100 % helium for 10 minutes prior to use and used to prepare the above solutions. These were further deoxygenated for 10 minutes before adding 200 æM $CoCl_2$ (2 ml) to 22.9 æM octapeptide (2ml). This mixture was again deoxygenated for 10 minutes prior to analysis by HPLC.

Octapeptide-cobalt complex (with oxygen): HPLC grade H_20 was bubbled with 100 % oxygen for 10 minutes prior to use and used to prepare the above solutions. These were further oxygenated for 10 minutes before adding 200 æM $CoCl_2$,(2 ml) to 22. æM octapeptide (2ml). This mixture was again oxygenated for 10 minutes prior to analysis by HPLC.

HPLC Analysis: Chromatography was carried out on a KS437 styrene / DVB polymer column (4.6 mm x 150 mm, pore diameter 100-150 A, BioDynamics) under isocratic conditions of 2 % acetonitrile in 30 mM Ammonium acetate pH 8.0 at a flow rate of 2 ml / min. Peaks were detected at 230 nm. Chromatography gave two distinct peaks at 230 nm, the first peak representing octapeptide-cobalt complex and

the second peak representing free octapeptide. Octapeptide-Co^{2+} complex formed in the presence of oxygen gave a higher ratio of complex over free peptide, as indicated by the first peak being the larger of the two. Octapeptide-Co^{2+} complex formed in the absence of oxygen again gave two peaks but the second peak was now the larger of the two, indicating less complex formation.

CONCLUSIONS: It would appear that oxygenated conditions enhance cobalt binding to the octapeptide.

EXAMPLE 18

The Effect of pH on the Octapeptide

OBJECTIVE: To optimize chromatography conditions for analysis of octapeptide by HPLC.

METHOD: The octapeptide was analyzed by HPLC using a KS437 styrene / DVB Polymer column (4.6 mm x 150 mm, pore diameter 100-150 A, 'BioDynamics) under isocratic conditions of 2 % acetonitrile in 30 mM Ammonium acetate at pH 6.2, 7.5 and 8.0 at a flow rate of 2 ml/min. Peaks were detected at 230 nm.

RESULTS: At pH 6.2, the octapeptide eluted after 1.6 min. At pH 8.0 the retention time had increased to 2.1 min. When the octapeptide was run at pH 7.5, two peaks were observed at 1.6 and 2.1 min.

CONCLUSIONS: The octapeptide exists in two forms depending on pH. The protonated form elutes at pH 6.2, and the deprotonated form at pH 8.0.

EXAMPLE 19

The Effect of pH on the Binding of Cobalt to the Octapeptide

OBJECTIVE: It was reported that the peptide peak 'shifted' when a solution of cobalt chloride was added to the octapeptide. It was decided to investigate this phenomenon fully as this would provide a direct tool for the determination of several parameters of cobalt binding to the octapeptide.

METHOD: 200 mM $CoCl_2$ (30 æl) was added to 2.3 mM octapeptide (270 æl), incubated at room temperature for 10 minutes and analyzed by HPLC. HPLC analysis: The octapeptide-cobalt complex was analyzed by HPLC using a KS437 styrene / DVB polymer column (4.6 mm x 150 mm, pore diameter 100-1 50 A, BioDynamics) under isocratic conditions of 2 % acetonitrile in 30 mM Ammonium acetate at pH 6.2 and 8.0 at a flow rate of 2 ml / min. Peaks were detected at 230 nm.

RESULTS: At pH 6.2, a single peak eluted after 1.6 min in the presence and absence of cobalt. At pH 8.0 however a single peak eluted after 1.2 min in the presence of cobalt and at 2.1 min in the absence of cobalt.

CONCLUSIONS: The octapeptide exists in two forms depending on pH. The protonated form that elutes at pH 6.2 is unable to bind cobalt and therefore its elution profile is unchanged. In contrast, the deprotonated form which exists at pH 8.0 is able to bind cobalt, resulting in an increased UV absorption and a decreased retention time, 1.2 min as opposed to 2.1 min for the free octapeptide.

EXAMPLE 20

The Titration of Octapeptide with Increasing Concentrations of Cobalt

OBJECTIVE: To determine whether increasing concentrations of cobalt resulted in a corresponding increase in octapeptide-cobalt complex formation.

METHOD: Octapeptide was used at a final concentration of 2.1 mM throughout, with increasing concentrations of $CoCl_2$, as shown in the Table below:

[$CoCl_2$](mM)	Vol $CoCl_2$ added (æl)	[Octapeptide] (mM)	Vol octapeptide added (æl)	Ratio of octapeptide: $CoCl_2$
0	0	2.3	27	1:0
1	3	2.3	27	21:1

1.25	3	2.3	27	16.8:1
2.25	3	2.3	27	9.3:1
4.5	3	2.3	27	4.7:1
10	3	2.3	27	2.1:1
18	3	2.3	27	1.2:1
36	3	2.3	27	1:1.7
72	3	2.3	27	1:3.4
200	3	2.3	27	1:9.5

HPLC analysis: The octapeptide-cobalt complex was analyzed by HPLC using a KS437 styrene/DVB polymer column (4.6 mm x 150 mm, pore diameter 100-150 A, BioDynamics) under isocratic conditions of 2 % acetonitrile in 30 mM Ammonium acetate at pH 8.0 at a flow rate of 2 ml/min. Peaks were detected at 230 nm.

RESULTS: Mean % Peak Height:

Final [$CoCl_2$] (mM)	Peak 1 (Octapeptide- Co complex)	Peak 2 (unknown)	Peak 3 (Octapeptide)
0	--	3.72	96.28
0.1	7.44	7.08	85.49
0.125	9.79	7.55	82.66
0.225	15.65	15.66	68.52
0.45	25.36	19.67	54.98
1.0	58.66	--	50.42
1.8	61.19	14.97	23.85
3.6	69.55	13.69	16.76
7.2	71.49	14.47	14.05
20.0	82.17	10.27	7.56

From the table immediately preceding, a plot of Log cobalt concentration versus % peak height for peak 3 was produced using Prism software. The 50 % binding constant as deduced from the exponential graph had a value of 0.6461 mM.

CONCLUSIONS: For 50 % binding, 0.6461 mM Co^{2+} binds to 2.1 mM octapeptide.

Therefore for 100 % binding, 1.2922 mM Co^{2+} binds to 2.1 mM octapeptide. The stoichiometry of cobalt binding to octapeptide is 0.615 cobalt to 1 octapeptide.

EXAMPLE 21

Liquid Chromatography-Mass Spectrometry of Octapeptide After the Addition of Cobalt

OBJECTIVE: To investigate whether mass spectral study would provide molecular weight information for the peptide and its corresponding cobalt complex.

METHOD: 200 mM $CoCl_2$ or H_20 (3 æl) was added to 2.3 mM octapeptide (27 µl) and incubated at room temperature for 10 minutes. LC-MS analysis: Liquid chromatography was performed using a KS437 styrene / DVB polymer column (4.6 mm x 150 mm, pore diameter 100-1 50 A, BioDynamics) under isocratic conditions of 2 % acetonitrile in 30 mM Ammonium acetate at pH 8.0 at a flow rate of 0.5 ml/min. Peaks were detected at 230 nm, and analyzed by on line mass spectrometry.

RESULTS: In the control sample, two molecular ion peaks were observed at 855.2 Da, representing the octapeptide alone, and at 877.2 Da, representing an octapeptide-sodium cluster. After the addition of 200 mM cobalt, one major peak was observed at 911.1 Da.

CONCLUSIONS: On addition of cobalt (59 Da) to the octapeptide, the molecular ion peak should occur at 914 Da. The actual peak occurs at 911 Da, representing the loss of protons.

EXAMPLE 22

Endprotease Lys-C Digest of Octapeptide and Its Subsequent Incubation with Cobalt

OBJECTIVE: Previous experiments confirm that $CoCl_2$ forms a stable complex with the octapeptide. In order to elucidate the site of attachment, the octapeptide was cleaved stereoselectively with the endoprotease Lys-C. The resultant

tetrapeptides upon incubation with $CoCl_2$ would allow elucidation of the probable binding site.

METHOD: Octapeptide 1.97 mg / ml (250 æl) was incubated with the endoprotease Lys-C 100 µg/ml (50 æl) at a substrate : enzyme ratio of 100 : 1 (w/w) in 8.3 mM Tricine, 1.6 mM EDTA pH 8.0 at 37° C for 24 h. After digestion, 27 æl of the product was incubated with 200 mM $CoCl_2$ (3 æl) at 20° C for 10 minutes prior to analysis by HPLC. HPLC Analysis: The products from the Lys-C digest were analyzed by HPLC using an amino column (4.6 mm x 250 mm, pore diameter 100 Å, BioDynamics-73) under isocratic conditions of 30 mM Ammonium acetate at pH 8.0 at a flow rate of 1.5 ml / min. Peaks were detected at 230 nm.

RESULTS: When the digested Lys-C products were run on HPLC, two peaks were observed at 2.6 and 8.9 min, designated tetrapeptides 1 and 2 respectively. Similarly after addition of cobalt to the digested products two peaks were again observed. However, tetrapeptide 1 exhibited an increased UV absorption and decreased retention time, eluting at 1.7 min as opposed to 2.6 min.

CONCLUSIONS: The octapeptide was digested at the C terminus of the lysine residue by the endoprotease yielding two tetrapeptides. On addition of cobalt to the endoprotease digested octapeptide, a single tetrapeptide-cobalt complex was formed with tetrapeptide 1. There appeared to be no effect on tetrapeptide 2.

EXAMPLE 23

Mass Spectrometry Analysis of the Tetrapeptide 1-Cobalt Complex

OBJECTIVE: To determine the identity of tetrapeptide 1.

EXPERIMENTAL: Tetrapeptides 1 and 2 were fractionated by HPLC and collected. $CoCl_2$ 1.2 mM (3 æl) was added to tetrapeptide 1 (27 æl) and incubated at room temperature for 10 minutes. Samples were subsequently run on MS as described previously.

RESULTS: Tetrapeptide 1 gave two molecular ion peaks at 470.1 and 477.1 Da. Tetrapeptide 2 gave a single peak at 404.0 Da. Tetrapeptide 1 -cobalt complex gave two peaks at 477.1 and 526 Da.

CONCLUSIONS: Tetrapeptide 1 is determined to be Asp-Ala-His-Lys with a molecular weight of 469 Da. Tetrapeptide 2 is determined to be Ser-Glu-Val-Ala (404 Da). Cobalt binds to Asp-Ala-His-Lys forming a complex of 526 Da with a loss of 3 protons. The molecular ion peak observed at 477.1 Da is a contaminant from the Lys-C preparation.

EXAMPLE 24

Manufacture of Calibrator Solutions

Human albumin solutions of 35 mg/ml containing cobalt of molar ratios of 0, 0.4, 0.625, 0.83, 1.25 and 2.5 to 1, cobalt:albumin, were made according to the following protocol.

An albumin solution of 35 mg/ml, Solution A, was made by initially dissolving 40 g solid human albumin (Fraction V, Sigma Chemical Co., St. Louis) in 900 ml 50 mM Tris-Cl, pH7.2, 0.15 NaCl, and assessing albumin concentration with bromo cresol green (BCG) assay (Sigma Chemical Co.). Additional buffer was added to produce an albumin concentration of 35 mg/ml. This solution was allowed to sit at 4°C for at least 24 hours prior to use.

To 500 ml of Solution A, 1.27 ml 0.32M $Co(OAc)_2 \cdot 6H_2O$ (160 mg Co salt/2 ml H_2O) (Sigma Chemical Co.) was added drop-wise with gentle swirling to produce a cobalt:albumin molar ratio of 1.25:1, Solution B. This solution was allowed to sit at room temperature for one hour prior to storage at 4°C until use.

Different volumes of Solutions A and B were mixed to produce additional calibrator solutions:

Cobalt:Albumin ratio	Solution A, ml	Solution B, ml
0	200	0
0.4	133	67
0.625	100	100
0.83	67	133
1.25	0	200

To make a cobalt:albumin calibrator solution of 2.5:1, 0.94 ml of 0.32M $Co(OAc)_2$ was added to 229 ml of Solution A. This solution was permitted to sit at room temperature for one hour and then stored at 4°C until use.

EXAMPLE 25

Quality Control Characterization of Calibrator Solutions

To obtain a cobalt:albumin ratio, one ml aliquots of each of the five calibrator solutions (each of which had been in storage for 24 hours prior to testing) was placed individually in dialysis bags and dialyzed against 400 ml 50mM Tris-Cl, pH7.2, 0.15M NaCl, with three changes of buffer at room temperature. Three to 5 µl of the dialyzates were withdrawn and analyzed for albumin using 1 ml of the BCG dye from Sigma Chemical Co. Absorbance was read at 628 nm after 30 seconds.

Cobalt was assessed by atomic absorption by Galbraith Laboratories, Inc., Knoxville, Tn.

The cobalt:albumin ratios were found to conform to expected values for all five calibrator solutions.

Added Cobalt, Co:albumin	At equilibrium, Co:albumin
0.4	0.16
0.625	0.26

0.83	0.31
1.25	0.46
2.50	0.74

These results indicate that the amount of cobalt bound per albumin molecule following dialysis remained proportional to the original metal concentration in the calibrator solution, indicating that the metal-cobalt complex is stable.

EXAMPLE 26

Generating a Standard Curve using Calibrator Solutions

Aliquots of 200 µl were withdrawn from each calibrator solution stored at 4°C into 12x75 mm borosilicate tubes and allowed to equilibrate to room temperature for at least 15 minutes.

A standard solution of 0.8% $CoCl_2 \cdot 6H_2O$ in H_20 had been made by dissolving 0.4 g solid in 500 ml deionized H_20 in a 500 ml polystyrene bottle; cobalt concentration was confirmed by atomic absorption by Galbraith Laboratories, Inc. Fifty µl of 0.8% $CoCl_2$ solution was added to each calibrator solution and gently mixed.

A 10mM DTT standard solution had been made by equilibrating the bottle of DTT (DL-dithiothreitol, Sigma Chemical Co.) to room temperature, weighing 12 mg and dissolving same in 8 ml deionized water. The sulfhydryl content of this solution was assessed using Ellman's Reagent, 5,5'-thio-bis(2-nitrobenzoic acid), Sigma Chemical Co. Exactly 10 minutes after addition of $CoCl_2$ solution to the calibrator solutions, 50 µl of the 10 mM DTT solution was added, mixed and allowed to react for 2 minutes. Substitution of DTT with 50 µl 0.9% NaCl was used as the blank. The reaction was quenched by the addition of 1.0 ml 0.9% NaCl. Absorbance at 470 nm on day 1 was read as soon as practicable. Absorbance was read again on days 12, 20 and 23:

Calibrator Co:albumin	A470 Day 1	A470 Day 12	A470 Day 20	A470 Day 23
0	0.26	0.26	0.23	0.27
0.4	0.32	0.30	0.28	0.29
0.625	0.33	0.33	0.31	0.31
1.25	0.39	0.40	0.37	0.37
2.5	0.64	0.60	0.60	0.57

Absorbance was plotted against metal concentration originally present in the calibrator solution. The plot was found to be substantially linear over the period studied.

EXAMPLE 27

The NMR Spectra for the Complex of Ni and Albumin N-terminal Amino Acids

Addition of cobalt or nickel chloride to the synthetic albumin N-terminus octapeptide afforded changes in the appearance of the ^{1}H-NMR spectrum for the resonances of the first three amino acid residues, with diagnostic changes of the Ala-2 methyl doublet at 1.35 ppm. Titration with $NiCl_2$ gave a sharp diamagnetic ^{1}H-NMR spectrum, while addition of $CoCl_2$ induced paramagnetism at the binding site resulting in significant broadening to the resonances associated with the three residues bound around the metal sphere. Figure 4 shows selected regions of the ^{1}H-NMR spectra (500 MHz, 10% D_2O in H_2O, 300K) showing the Ala resonances (Ala-2 and Ala-8) of the octapeptide (A) free of any metal, with a Lys-4 methylene resonance appearing between the doublets for Ala2 at about 1.35 ppm and for Ala8 at about 1.4, (B) with 0.5 equiv. of $NiCl_2$ added resulting in a shift of the Ni-bound Ala2 doublet to about 1.3, (C) with 1.0 equiv. of $NiCl_2$ added, (D) with 0.5 equiv. of $CoCl_2$ added, and (e) with 1.0 equiv. of $CoCl_2$ added. In all cases, the appearance and chemical shift of the resonances attributed to Ser-5, Glu-6, Val-7 and Ala-8 did not change significantly

after metal addition (up to one equivalent). All these observations were conserved in metal titration experiments with the synthetic tetrapeptide (N-Asp-Ala-His-Lys).

EXAMPLE 28

U.V. Spectroscopic Evidence of Co Binding to Albumin Pep-12 Peptides

The albumin N-terminal peptide Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys- (Pep 12), was synthesized by Quality Controlled Biochemicals, Inc. both in N-acetylated-Asp and free Asp forms, each with free C-terminus. Solutions of 1 mg/ml of the two peptides were made in Tris 50 mM 0.9% NaCl pH7.2 and analyzed by UV spectroscopy (Ocean Optics SD 2000 and AIS Model DT 1000 as light source). U.V. spectra of Pep-12 and acetylated Pep-12 are set forth in Figs. 5A and 5B, respectively. Addition of $CoCl_2 \cdot 6H_2O$ 0.8% (20 µL of the peptide solution) shows a dramatic shift of the λ maximum of the peptide peak as well as a major increase in the extinction coefficient for the nonacetylated Pep-12 (Fig. 6A) and no change in the spectrum of the acetylated Pep-12 (Fig. 6B).

Solutions of Pep-12 and acetylated Pep-12 were made into solutions of 1 mg/ml in Tris 50 mM NaCl 0.9% pH7.2. Five mixtures of the two starting peptides were made: 100% Pep-12, 75:25 Pep-12:AcPep-12, 50:50 Pep-12:AcPep-12, 25:75 Pep-12:AcPep-12 and 100% AcPep-12.

	1	2	3	4	5
Pep-12 1 mg/ml	20 ml	15	10	5	0
AcPep-12 1 mg/ml	-	5	10	15	20
+/- CoCl2 0.08%	20	20	20	20	20

Spectral analysis of solutions 1-5 is represented in Fig. 7, from which it can be seen that Pep-12 binds cobalt, AcPep-12 does not bind cobalt. Further, as acetylation increases, cobalt binding goes down.

EXAMPLE 29

U.V. Spectroscopic Evidence of Co Binding to Albumin Pep-10

Pep-10 was made into 1 mg/ml solutions and incubated with $CoCl_2$ (0.08%). Spectral scans were obtained (data not shown). There was no apparent difference in the absorbance after addition of cobalt, indicating that Pep-10 does not bind cobalt.

EXAMPLE 30

Copper/Cobalt Competition Binding for Albumin Pep-12

Pep-12 (20 μL of 1 mg/ml or 0.014 μMol) was mixed with 5 μL $CuCl_2$ (0.08% or 0.023 μMol) and 20 μL $CoCl_2$ 0.08% (0.067 μMol). The U.V. spectral curve is shown in Fig. 8A. AcPep-12 (20 μL of 1 mg/ml or 0.014 μMol) was also mixed with 5 μL $CuCl_2$ (0.08% or 0.023 μMol) and 20 μL $CoCl_2$ 0.08% (0.067 μMol). The U.V. spectral curve is shown in Fig. 8B. The $CuCl_2$ was added to Pep-12 and AcPep-12 before addition of $CoCl_2$. No shift or change occurred by this manipulation.

Pep-12 binds copper and cannot therefore display a shift and increase absorbance when cobalt is added. The tails appearing on the peaks in Figs. 8A and 8B are due to absorbance of copper in the U.V. range.

EXAMPLE 31

Enzymatic Acetylation of N-Terminal Pep-8 and Human Serum Albumin

Human serum albumin (Sigma A-1653) was incubated at 37°C for 1 h with N-acetyl transferase and acetyl CoA, and spectral scans were obtained at various times (2-60 minutes). A steady increase at A235 was observed (assuming A235 reflects acetylation), reaching a plateau at about 40 minutes (data not shown).

Likewise, Pep-8 (Asp-Ala-His-Lys-Ser-Glu-Val-Ala), was acetylated according to the following conditions:

	1	2	3	4	5	6	7	8
Pep-8	250 μL	250 μL	250 μL	250 μL				
NAT	50 μL			50 μL		50 μL		50 μL

AcCoA	25 μL	25 μL			25 μL			25 μL
Buffer		50 μL	75 μL	25 μL	300 μL	275 μL	325 μL	250 μL
$CoCl_2$	+/- 50 μL	+/- 50 μL	+/- 50 μL	+/- 50 μL	+/- 50 μL	+/- 50 μL	+/- 50 μL	+/- 50 μL

The Pep-8 was 1 mg/ml in a solution of Tris 50 mM, pH 7.5, 0.15 NaCl. The N-acetyl-transferase was 10 U/mL (Sigma A426). The acetyl CoA was 10 mg/ml in H_2O (Sigma A2056). The Buffer was Tris 50 mM, pH 7.5, 0.15 NaCl. After completion of the reaction, test tubes were centrifuged using Centricon (3000 MW cutoff) to remove N-acetyl transferase and acetyl CoA which introduce interference in the U.V. range. The +/- in the final row refers to the fact that the absorbance at 235 was measured with and without addition of $CoCl_2$. Addition of cobalt did not result in a shift of the peak, indicating that the acetylated Pep-8 did not bind cobalt.

Fig. 9 is the subtracted scan of the centrifuged acetylated Pep-8, plus reaction mixture and cobalt, minus the reaction mixture without the cobalt, showing a peak at about 280 nm, presumably the acetylated Pep-8.

EXAMPLE 32

Confirmation of Ni, Co and Co Binding to Modified Peptides by ^{1}H-NMR (800 MHz)

Peptide I: The N-terminal dodecapeptide, Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys.

The N-terminal dodecapeptide was titrated with each of cobalt, copper and nickel. The methyl signals of the two Ala residues (positions 2 and 8) appear at the same resonance, namely 1.3 ppm. Fig. 10A is Peptide 1 at pH 2.55 with no metal. Fig. 10B is Peptide 1 at pH 7.33 with no metal. Titration with 0.3 equivalent $NiCl_2$ at pH 7.30 is characterized by the appearance of a set of peaks at 1.25 ppm which is characteristic of the methyl of Ala at position 2 (Fig. 10C). After the addition of one equivalent of $NiCl_2$ at pH 7.33, the methyl groups of Ala at positions 2 (1.3 ppm) and 8 (1.25 ppm) are equivalent, showing that the metal binds and that the binding is stoichiometric (Fig. 10D). Fig. 10 scans were conducted at 800 MHz, 10% D_20/90%H_20 (Ala-Me region).

The addition of $CoCl_2$ also shows binding but the peaks are broader with a shift in the methyl group Ala 2 to 1.7 ppm (Fig. 11). Fig. 11A shows Peptide 1's Ala2 and Ala8 methyl signals at 1.3 (pH 2.56). Fig. 11B shows Peptide 1 at pH 7.45. Fig. 11C shows widening of the 1.3 ppm peak as 0.5 equivalent $CoCl_2$ is added at pH 7.11. Fig. 11D shows a separate peak for Ala2-Me at 1.7 ppm with 1.0 equivalent $CoCl_2$ at pH 7.68. Fig. 11 scans were conducted at 500 MHz, 10% D_20/90% H_2O (Ala-Me region).

The addition of $CuSO_4$ causes even more broadening of both methyl groups at positions 2 and 8 to the point where, after addition of 1 equivalent of $CuSO_4$, both signals are lost (Fig. 12). Fig. 12A shows Peptide 1 at pH 2.56 with Ala2 and Ala8 methyl signals at 1.35 ppm. Fig. 12B shows Peptide 1 at pH 7.54. Fig. 12C shows Peptide 1 with a broadening of the signal at 1.35 ppm, due to about 0.5 equivalent $CuSO_4$ (pH 7.24). Fig. 12D shows Peptide 1 with about 1 equivalent $CuSO_4$ at pH 7.27. Fig. 12 scans were conducted at 500 MHz, 10% D_20/90% H_2O (Ala-Me region).

Peptide 2: The N-Terminal dodecapeptide, Asp-Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys, in which the amino group of the N-terminal Asp has been acetylated.

Addition of $NiCl_2$ to the acetylated derivative does not result in binding, i.e., there is no appearance of additional peaks (Fig. 13). However, addition of even one equivalent of $NiCl_2$ broadens the spectrum considerably due to the fact that the nickel is free in solution. Fig. 13A shows Peptide 2 at pH 2.63 with the Ala2 and Ala8 Me signals at about 1.28 ppm. Fig. 13B shows Peptide 2 at pH 7.36. Fig. 13C shows Peptide 2 with about 0.5 equivalent $NiCl_2$ at pH 7.09. Fig. 13D shows Peptide 2 with about 1 equivalent $NiCl_2$ at pH 7.20. Fig. 13 scans were conducted at 800 MHz, 10% D_20/90% H_20 (Ala-Me region).

Peptide 3: The N-Terminal Unodecapeptide, Ala-His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys, in which the terminal Asp is missing.

The N-terminal residue is Ala and consequently the position of the doublet from the methyl group is pH dependent (Fig 14). Addition of $NiCl_2$ does not result in complex formation. Fig. 14A shows Peptide 3 at pH 2.83 with the Ala2 signal at 1.5 and the Ala8 signal at 1.3. Fig. 14B shows Peptide 3 at pH 7.15. Fig. 14C shows Peptide 3 with 0.13 equivalent $NiCl_2$ at pH 7.28. Fig. 14D shows Peptide 3 with about 0.25 equivalent $NiCl_2$ at pH 7.80. Fig. 14E shows Peptide 3 with 0.5 equivalent $NiCl_2$ at pH 8.30. Fig. 14 scans were conducted at 500 MHz, 10% D_20/90% H_2O (Ala-Me region).

Peptide 4: The N-Terminal decapeptide, His-Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys, in which Asp-Ala has been removed.

Upon addition of $NiCl_2$ the spectrum broadens unrecognizably with no evidence of binding (Fig. 15). Fig. 15A shows Peptide 4 with an Ala8 signal at 1.8 ppm at pH 2.72. Fig. 15B shows Peptide 4 at pH 7.30. Fig. 15C shows Peptide 4 with 0.5 equivalent $NiCl_2$, pH 8.30. Fig. 15D shows Peptide 4 with about 1 equivalent $NiCl_2$ at pH 8.10. Fig. 15 scans were conducted at 800 MHz, 10% D_20/90% H_2O (Ala-Me region).

Peptide 5: The nonpeptide, Lys-Ser-Glu-Val-Ala-His-Arg-Phe-Lys, in which the tripeptide Asp-Ala-His is missing.

Again there is not much change in the spectrum after addition of 0.3 equivalents of $NiCl_2$ (Fig. 16C) except for the decrease in peak intensity and peak broadening upon addition of less than 1 equivalent of metal ions (Fig. 16D). There is no evidence of metal binding. Fig. 16A is Peptide 5 at pH 2.90 with the Ala8 signal at 1.3 ppm. Fig. 16B is Peptide 5 at pH 7.19. Fig. 16C is Peptide 5 with 0.3 equivalent $NiCl_2$, pH 7.02. Fig. 16D is Peptide 5 with about 0.6 equivalent $NiCl_2$ at pH 7.02. Fig. 16 scans were conducted at 500 MHz, 10% D_2O/90% H_2O (Ala-Me region).

Peptide 6: The N-terminal tetrapeptide, Asp-Ala-His-Lys.

The addition of $NiCl_2$ (Fig. 17), $CoCl_2$ (Fig. 18) and $CuSO_4$ (Fig. 19) all gave diagnostic changes consistent with metal ion binding. The spectra resemble those obtained with the dodecapeptide (Peptide 1) and not those obtained with Peptides 2, 3, 4 and 5.

Fig. 17A is the N-terminal tetrapeptide at pH 2.49 with an Ala2 signal at 1.3 ppm. Fig. 17B is the tetrapeptide at pH 7.44. Fig. 17C is the tetrapeptide with about 0.8 equivalent $NiCl_2$ at pH 7.42. Fig. 17D is the tetrapeptide with about 1 equivalent $NiCl_2$ at pH 7.80.

Fig. 18A is the tetrapeptide at pH 7.44 with the Ala2 peak at 1.3 ppm. Fig. 18B is the tetrapeptide with about 0.3 equivalent $CoCl_2$ at pH 7.23. Fig. 18C is the tetrapeptide with about 0.8 equivalent $CoCl_2$ at pH 7.33.

Fig. 19A is the tetrapeptide at pH 7.31 with the Ala2 signal at 1.3 ppm. Fig. 19B is the tetrapeptide with about 0.5 equivalent $CuSO_4$ at pH 7.26. Fig. 19C is the tetrapeptide with about 1.0 equivalent $CuSO_4$ at pH 7.32.

Figs. 17-19 scans were conducted at 800 MHz, 10% H_20/90% D_2O (Ala-Me region).

* * * * *

The above description of the invention is intended to be illustrative and not limiting. Various changes or modification in the embodiments described may occur to those skilled in the art. These can be made without departing from the spirit or scope of the invention.

What is claimed is:

1. A method for detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of:

(a) contacting a biological sample containing albumin from said patient with an excess quantity of a metal ion salt, whereby said metal ion binds to the N-terminus of naturally occurring human albumin, to form a mixture containing bound metal ions and unbound metal ions,

(b) determining the amount of metal ions bound to the albumin N-terminus, and

(c) correlating the amount of bound metal ions to a known value to determine the occurrence or non-occurrence of an ischemic event.

2. The method of claim 1, wherein said sample is serum or plasma.

3. The method of claim 1, wherein the sample is purified albumin.

4. The method of claim 1, wherein said metal ion salt is a salt of a metal selected from the group consisting of V, As, Co, Cu, Sb, Cr, Mo, Mn, Ba, Zn, Ni, Hg, Cd, Fe, Pb, Au and Ag.

5. The method of claim 1, wherein said metal ion is cobalt.

6. The method of claim 1, wherein step (b) is conducted using atomic absorption spectroscopy, atomic emission spectroscopy or an immunological assay.

7. The method of claim 6, wherein said immunological assay is conducted using an antibody specific to an antigen comprising the compound Asp-Ala-His-Lys-R, wherein R is said metal ion.

8. The method of claim 6, wherein said immunological assay is conducted using an antibody to a human serum albumin-metal complex.

9. A method of detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of:

(a) contacting a biological sample containing albumin from said patient with a predetermined excess quantity of a salt of a metal selected from the group consisting of V, As, Co, Cu, Sb, Cr, Mo, Mn, Ba, Zn, Ni, Hg, Cd, Fe, Pb, Au and Ag, to form a mixture containing metal ions bound to the N-terminus of albumin and unbound metal ions,

(b) contacting said mixture with an aqueous color forming compound solution to form a colored solution, wherein said compound forms color when bound to said unbound metal ion,

(c) determining the color intensity of said colored solution to detect the presence of unbound metal ions to provide a measure of bound metal ions, and

(d) correlating the amount of bound metal ions to a known value to determine the occurrence or non-occurrence of an ischemic event.

10. The method of claim 9, wherein said aqueous color forming compound comprises the compound Asp-Ala-His-Lys-R, wherein R is any group capable of forming color when bound to said metal ion.

11. The method of claim 9, wherein said sample is serum or plasma.

12. The method of claim 9, wherein said sample is purified albumin.

13. The method of claim 9, wherein said metal ion salt is a salt of cobalt.

14. A method for ruling-out the existence of ischemia in a patient, wherein said patient possesses one or more cardiac risk factors, comprising

(a) application of the method of claim 1 or 9 to said patient;

(b) subjecting said patient to an exercise treadmill test followed by a second application of the same method of claim 1 or 9;

(b) comparing the results of the two applications of said method.

15. A method for evaluation of a patient presenting with angina or angina-like symptoms to detect the occurrence or non-occurrence of a myocardial infarction, comprising

(a) application of the method of claim 1 or 9,

(b) application of an electrocardiographic test,

(c) correlating the results of step (a) with the results of said electrocardiographic test to determine the occurrence or non-occurrence of a myocardial infarction.

16. A method for supplementing electrocardiographic results to determine the occurrence or non-occurrence of an ischemic event, comprising

(a) application of the method of claim 1 or 9,

(b) application of an electrocardiographic test, and

(c) correlating the results of step (a) with the results of said electrocardiographic test to determine the occurrence or non-occurrence of an ischemic event.

17. A method for comparing levels of ischemia in patients at rest and during exercise, comprising application of the following steps at designated times:

(a) application of the method of claim 1 or 9 at a first designated time,

(b) administration of an exercise treadmill test followed by a second application of the same method employed in step (a),

(c) comparing the results of step (a) with the results obtained in step (b), and

(d) repeating steps (a) and (b) at additional designated times,

wherein results obtained designated at each designated time are compared.

18. The method of claim 17, wherein said designated times are three months, six months and one year.

19. A method for detecting the occurrence or non-occurrence of an ischemic event in a patient comprising the steps of:

(a) detecting the amount of endogenous copper ions present in a purified albumin sample from said patient, and

(b) correlating the quantity of copper ions present with a known value to determine the occurrence or non-occurrence of an ischemic event.

20. The method of claim 19, wherein said detecting step is conducted using atomic absorption spectroscopy, atomic emission spectroscopy or an immunological assay.

21. The method of claim 20, wherein said immunological assay is conducted using an antibody specific to an antigen comprising the compound Asp-Ala-His-Lys-R, wherein R is copper.

22. The method of claim 20, wherein said immunological assay is conducted using an antibody to a human serum albumin-copper complex.

23. A method for ruling-out the existence of ischemia in a patient, wherein said patient possesses one or more cardiac risk factors, comprising:

(a) applying the method of claim 19 to the patient,

(b) subjecting said patient to an exercise treadmill test followed by a second application of the method of claim 19;

(b) comparing the results of the two applications of the method of claim 19.

24. A method for evaluation of a patient presenting with angina or angina-like symptoms to detect the occurrence or non-occurrence of a myocardial infarction, comprising

(a) application of the method of claim 19,

(b) application of an electrocardiographic test,

(c) correlating the results of application of the method of claim 19 with the results of said electrocardiographic test to determine the occurrence or non-occurrence of a myocardial infarction.

25. A method for supplementing electrocardiographic results to determine the occurrence or non-occurrence of an ischemic event, comprising

(a) application of the method of claim 19,

(b) application of an electrocardiographic test, and

(c) correlating the results of application of the method of claim 19 with the results of said electrocardiographic test to determine the occurrence or non-occurrence of an ischemic event.

26. A method for comparing levels of ischemia in patients at rest and during exercise, comprising application of the following steps at designated times:

(a) application of the method of claim 19 at a first designated time,

(b) administration of an exercise treadmill test followed by a second application of the method of claim 19,

(c) comparing the results of the application of the method of claim 19 prior to administration of the exercise treadmill test with the results of the application of the method of claim 19 after administration of the exercise treadmill test, and

(d) repeating steps (a) and (b) at additional designated times,

wherein results obtained at said designated times are compared.

27. The method of claim 26, wherein said designated times are three months, six months and one year.

28. A method of detecting or measuring an ischemic event in a patient comprising:

(a) contacting a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives with an excess quantity of metal ion that binds to the N-terminus of naturally-occurring albumin, whereby albumin-metal complexes are formed,

(b) partitioning the complexes from said derivatives, if any,

(c) measuring at least one of said derivatives, if any, and

(d) comparing said measured derivative to a known value, whereby the ischemic event may be detected or measured.

29. The method of claim 28 wherein said metal is Ni or Co.

30. The method of claim 28 wherein said metal of step (a) is bound to a solid support and said partitioning step (b) comprises separating said derivatives from the solid support to which the metal is bound.

31. The method of claim 28 wherein said metal of step (a) is in solution and said partitioning step (b) comprises contacting said complexes with an antibody to the albumin-metal complex, said antibody being bound to a solid support.

32. The method of claim 28 wherein said measuring step (c) comprises contacting said derivative with an antibody to the derivative.

33. A method for detecting or measuring an ischemic event in a patient comprising:

(a) contacting a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives with an excess of a metal ion, whereby a albumin-metal complex is formed,

(b) contacting the mixture of step (a) with an antibody to said complex, said antibody being bound to a solid support,

(c) separating the complex from said N-terminal derivatives, if any,

(d) measuring the amount of at least one N-terminal derivative, if any, and

(e) comparing the measured N-terminal derivative to a known value, whereby an ischemic event may be detected or measured.

34. The method of claim 33, wherein the metal ion is cobalt ion.

35. The method of claim 33, wherein said measuring step (d) comprises contacting the derivative with an antibody.

36. An immunoassay diagnostic kit for an ischemic event comprising:

an excess quantity of a metal ion to mix with a patient sample which comprises naturally-occurring albumin and optionally albumin N-terminal derivatives, said naturally-occurring albumin forming a complex with said metal ion,

a first elongated solid support having a first and a second end, said first end having a filter for application of said patient sample mixture, an area of immobilized antibody to said albumin-metal complex between the first end the second end, and an area of immobilized ligand to albumin proximate the second end,

whereby after application of said mixture of patient sample and metal ion to said filter, said albumin-metal complex is immobilized at said area of immobilized antibody, and said albumin N-terminal derivatives migrate and bind to the albumin ligand proximate the second end.

37. The kit of claim 36, wherein said metal ion is cobalt ion.

38. The kit of claim 36, further comprising an end of process indicator at the second end of said solid support.

39. The kit of claim 36, further comprising a second elongated solid support having a first and second end, said second support first end sharing said filter for application of said patient sample mixture with said first elongated support, and having an area of immobilized ligand to albumin between the first and second ends, said second support serving as a control.

40. The kit of claim 39, further comprising an end of process indicator at the second end of said second solid support.

41. An immunoassay diagnostic kit for an ischemic event comprising:

a circular solid support comprising an interior filter circle surrounded by an inner concentric ring and an outer concentric ring, wherein

said inner filter circle is for application of a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives, said sample having been mixed with an excess quantity of a metal ion, whereby an albumin-metal complex has been formed,

said inner concentric ring is divided into a first and second half, said first half containing a ligand to said albumin-metal complex, and

said outer concentric ring is divided into a first and second half, each said outer ring halves aligned with the inner ring halves, and each said outer ring halves containing ligands to a non N-terminus epitope of naturally-occurring albumin and to albumin N-terminal derivatives.

42. An immunoassay diagnostic kit for an ischemic event comprising:

a circular solid support comprising an inner filter circle surrounded by a concentric ring, wherein

said inner filter circle is for application of a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives, said sample having been mixed with an excess quantity of a metal ion, whereby an albumin-metal complex has been formed, and

said concentric ring is divided into a first and a second half, said first half having a ligand to the albumin-metal complex, and the second half having ligands to a non N-terminus epitope of naturally-occurring albumin and to albumin N-terminal derivatives.

43. A method of detecting or measuring an ischemic event in a patient comprising:

(a) contacting a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives with an excess quantity of a metal ion bound to a solid support, whereby the metal ion binds to the N-terminus of naturally-occurring albumin, forming albumin-metal complexes,

(b) separating the complexes from said derivatives, if any,

(c) measuring at least one of said derivatives, if any, and

(d) comparing said measured derivative to known value, whereby the ischemic event may be detected or measured.

44. The method of claim 43 wherein the metal ion is nickel ion.

45. The method of claim 43 wherein the solid support is a diacetate or a phosphonate matrix.

46. The method of claim 43 wherein said measuring step (c) comprises contacting said derivative with an antibody to the derivative.

47. A metal affinity diagnostic kit for an ischemic event comprising:

a first elongated solid support having a first and a second end, said first end having a filter for application of a patient sample, an area of immobilized metal ion between the first and the second end, and an area of immobilized ligand to naturally-occurring albumin or albumin N-terminal derivatives proximate the second end.

48. The kit of claim 47, wherein said immobilized metal is nickel.

49. The kit of claim 47, further comprising an end of process indicator at the second end of said first solid support.

50. The kit of claim 47, further comprising a second elongated solid support having a first and second end, said second support first end sharing said filter for application of said patient sample with said first solid support, and having an area of immobilized ligand to naturally-occurring albumin and albumin N-terminal derivatives proximate the second end, said second support serving as a control.

51. The kit of claim 50, further comprising and end of process indicator at the second end of said second solid support.

52. A monoclonal antibody directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the four N-terminal amino acids of SEQ. ID. NO. 1.

53. A monoclonal antibody directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the three N-terminal amino acids of SEQ. ID. NO. 1.

54. A monoclonal antibody directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the two N-terminal amino acids of SEQ. ID. NO. 1.

55. A monoclonal antibody directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the N-terminal amino acid of SEQ. ID NO. 1.

56. A monoclonal antibody directed to an epitope at the N-terminus of SEQ. ID NO. 2.

57. A calibrator composition comprising a predetermined molar ratio of naturally-occurring albumin and a metal that complexes to the N-terminus of said albumin, whereby complexed albumin and unbound albumin form when said composition is in aqueous solution, wherein said ratio is between 0.1:1 and 1:0.1.

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58. The composition of claim 57 wherein said metal is selected from the group consisting of Cu, Ni and Co.

59. The composition of claim 57 wherein the predetermined ratio of albumin to
10 metal is 3:1.

60. The composition of claim 57 wherein the predetermined ratio of albumin to metal is 1:3.

15 61. The composition of claim 57 wherein the predetermined ratio of albumin to metal is 1:1.

62. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 1, comprising the step of:

20 applying the calibrator solution of claim 57 to the analyzer to determine the amount of metal ions bound to the albumin N-terminus, whereby the predetermined ratio of albumin to metal serves as a standard for calibration.

25

63. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 15, comprising the steps of:

(a) mixing the calibrator composition solution of claim 57 with a predetermined amount of an excess metal salt, whereby said unbound albumin binds to said excess metal ion, generating unbound metal ions,

(b) contacting the mixture of step (a) with color forming solution to form a colored solution,

(c) applying the mixture of step (b) to the analyzer, whereby the predetermined ratio of albumin to metal serves as a standard for calibration.

64. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 19, comprising the step of:

applying the calibrator solution of claim 57 wherein the metal is copper to the analyzer to determine the amount of copper ions bound to the albumin N-terminus, whereby the predetermined ratio of albumin to copper serves as a standard for calibration.

AMENDED CLAIMS

[received by the International Bureau on 29 February 2000 (29.02.00);
original claims 52-56 and 63 amended; new claims 65-76 added;
remaining claims unchanged (4 pages)]

50. The kit of claim 47, further comprising a second elongated solid support having a first and second end, said second support first end sharing said filter for application of said patient sample with said first solid support, and having an area of immobilized ligand to naturally-occurring albumin and albumin N-terminal derivatives proximate the second end, said second support serving as a control.

51. The kit of claim 50, further comprising and end of process indicator at the second end of said second solid support.

52. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the four N-terminal amino acids of SEQ. ID. NO. 1.

53. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the three N-terminal amino acids of SEQ. ID. NO. 1.

54. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the two N-terminal amino acids of SEQ. ID. NO. 1.

55. A ligand directed to an epitope at the N-terminus of the albumin N-terminal derivative which lacks the N-terminal amino acid of SEQ. ID NO. 1.

56. A ligand to an epitope at the N-terminus of SEQ. ID NO. 2.

63. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 9, comprising the steps of:

(a) mixing the calibrator composition solution of claim 57 with a predetermined amount of an excess metal salt, whereby said unbound albumin binds to said excess metal ion, generating unbound metal ions,

(b) contacting the mixture of step (a) with color forming compound to form a colored solution, and

(c) applying the mixture of step (b) to the analyzer, whereby the predetermined ratio of albumin to metal serves as a standard for calibration.

64. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 19, comprising the step of:

applying the calibrator solution of claim 57 wherein the metal is copper to the analyzer to determine the amount of copper ions bound to the albumin N-terminus, whereby the predetermined ratio of albumin to copper serves as a standard for calibration.

65. A method of detecting an albumin N-terminal derivative which lacks four N-terminal amino acids of SEQ. ID. NO. 1, comprising contacting a sample comprising said derivative with the ligand of claim 52.

66. A method of detecting an albumin N-terminal derivative which lacks three N-terminal amino acids of SEQ. ID. NO. 1, comprising contacting a sample comprising said derivative with the ligand of claim 53.

67. A method of detecting an albumin N-terminal derivative which lacks two N-terminal amino acids of SEQ. ID. NO. 1, comprising contacting a sample comprising said derivative with the ligand of claim 54.

68. A method of detecting an albumin N-terminal derivative which lacks an N-terminal amino acid of SEQ. ID NO. 1, comprising contacting a sample comprising said derivative with the ligand of claim 55.

69. A method of detecting an albumin N-terminal derivative which is acetylated at its N-terminal Asp residue (SEQ. ID. NO. 2), comprising contacting a sample comprising said derivative with the ligand of claim 56.

70. A diagnostic kit for an ischemic event comprising:

a circular solid support comprising an interior filter circle surrounded by an inner concentric ring and an outer concentric ring, wherein

said inner filter circle is for application of a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives,

said inner concentric ring is divided into a first and second half, said first half containing an excess amount of bound metal ion to bind to the N-terminus of said naturally-occurring albumin, and

said outer concentric ring is divided into a first and second half, each said outer ring halves aligned with the inner ring halves, and each said outer ring halves containing ligands to a non-N-terminus epitope of naturally occurring albumin and to albumin N-terminal derivatives.

71. A diagnostic kit for an ischemic event comprising:

a circular solid support comprising an inner filter circle surrounded by a concentric ring, wherein

said inner filter circle is for application of a patient sample comprising naturally-occurring albumin and optionally albumin N-terminal derivatives,

said concentric ring is divided into a first and second half, said first half having an excess amount of bound metal to bind to the N-terminus of naturally-occurring albumin, and the second half having ligands to a non-N-terminus epitope of naturally-occurring albumin and to albumin N-terminal derivatives.

72. A calibrator composition comprising a predetermined molar ratio of naturally-occurring albumin and albumin N-terminal derivatives, wherein said ratio is between 0.1:1 and 1:0.1.

73. The calibrator composition of claim 72 wherein the molar ratio of naturally-occurring albumin to albumin N-terminal derivatives is 3:1.

74. The calibrator composition of claim 72 wherein the molar ratio of naturally-occurring albumin to albumin N-terminal derivatives is 1:3.

75. The calibrator composition of claim 72 wherein the molar ratio of naturally-occurring albumin to albumin N-terminal derivatives is 1:1.

76. A method of calibrating an analyzer that detects or measures an ischemic event according to the method of claim 9 comprising the steps of:

(a) mixing the calibrator composition of claim 72 with a predetermined amount of an excess metal salt, whereby naturally-occurring albumin binds to said excess metal ion, generating unbound metal ions,

(b) contacting the mixture of step (a) with a color forming compound to form a colored solution,

(c) applying the mixture of step (b) to the analyzer, whereby the predetermined ratio of naturally-occurring albumin to albumin N-terminal derivatives serves as a standard for calibration.

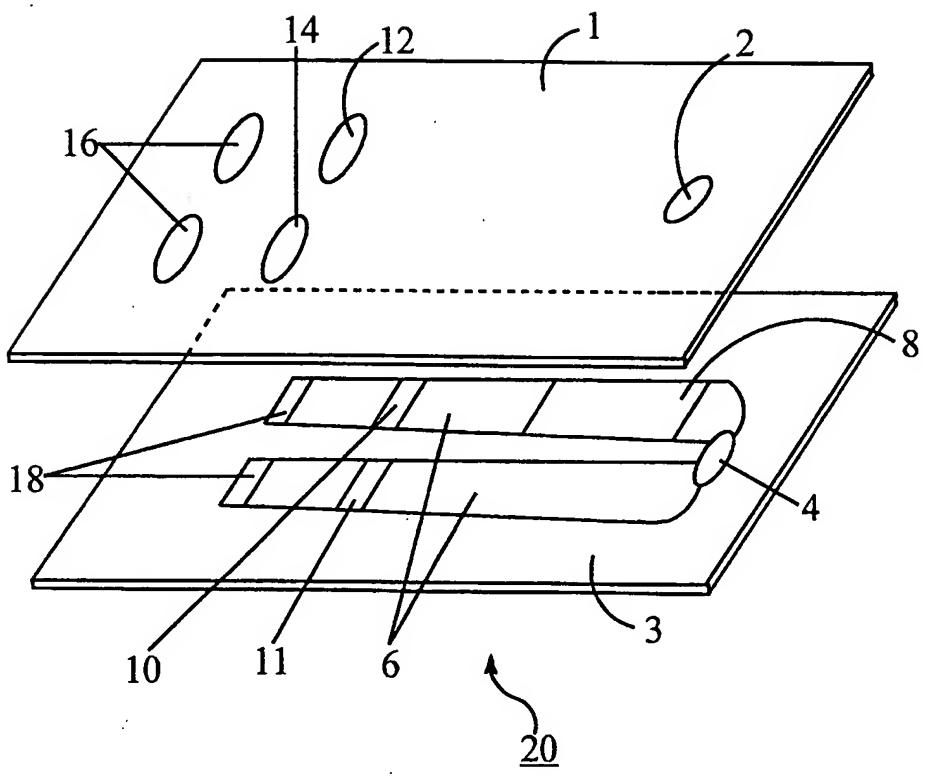


Fig. 1

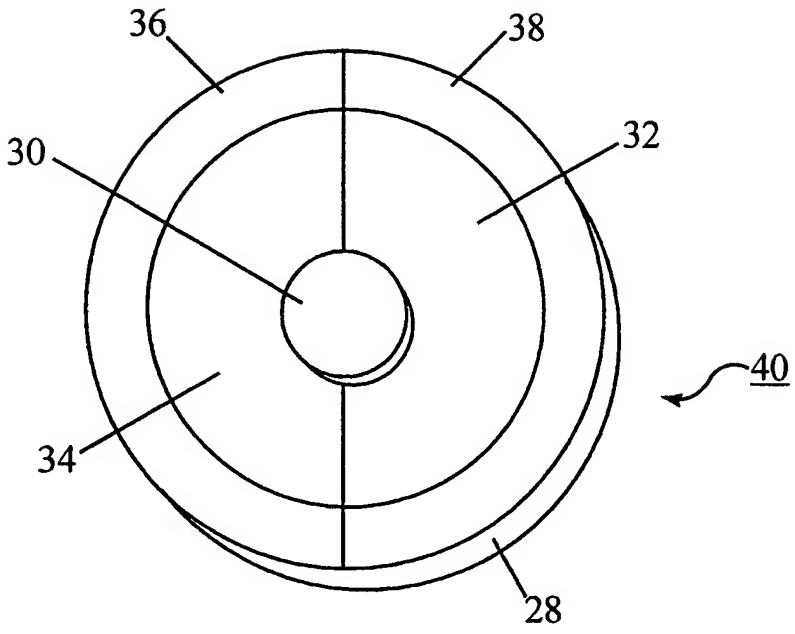


Fig. 2

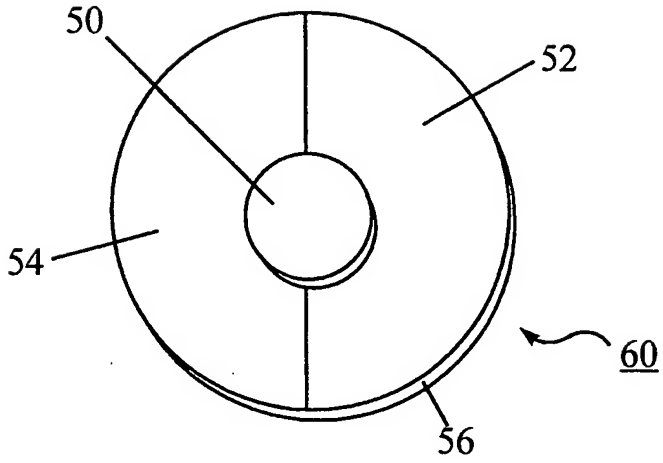


Fig. 3

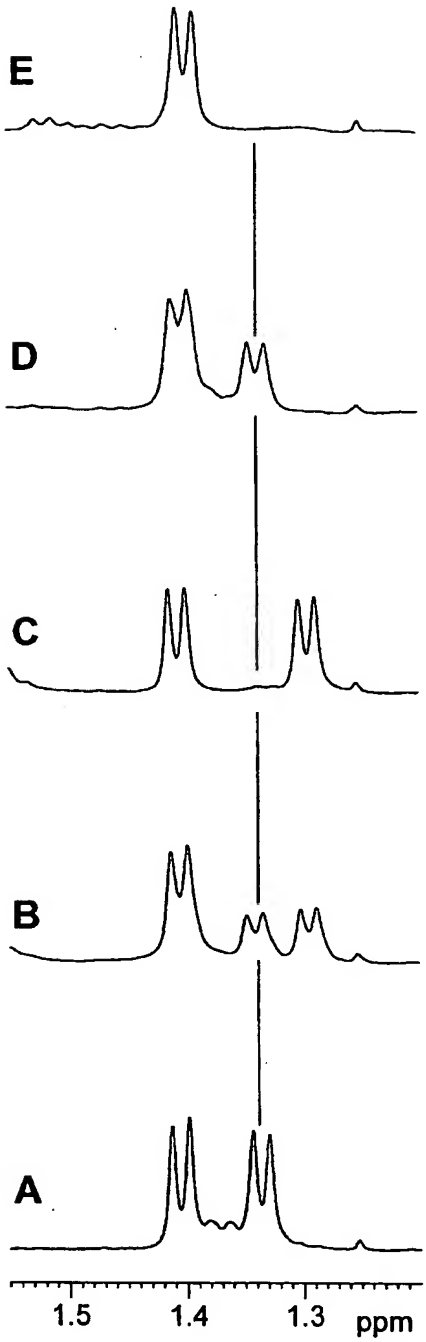


Figure 4

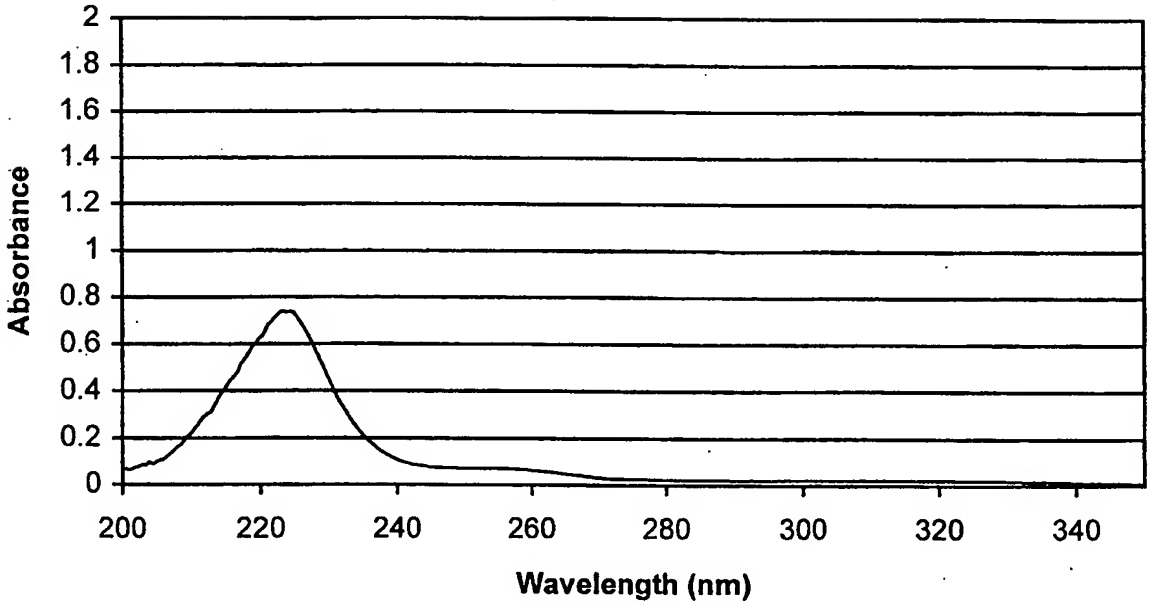


Figure 5A

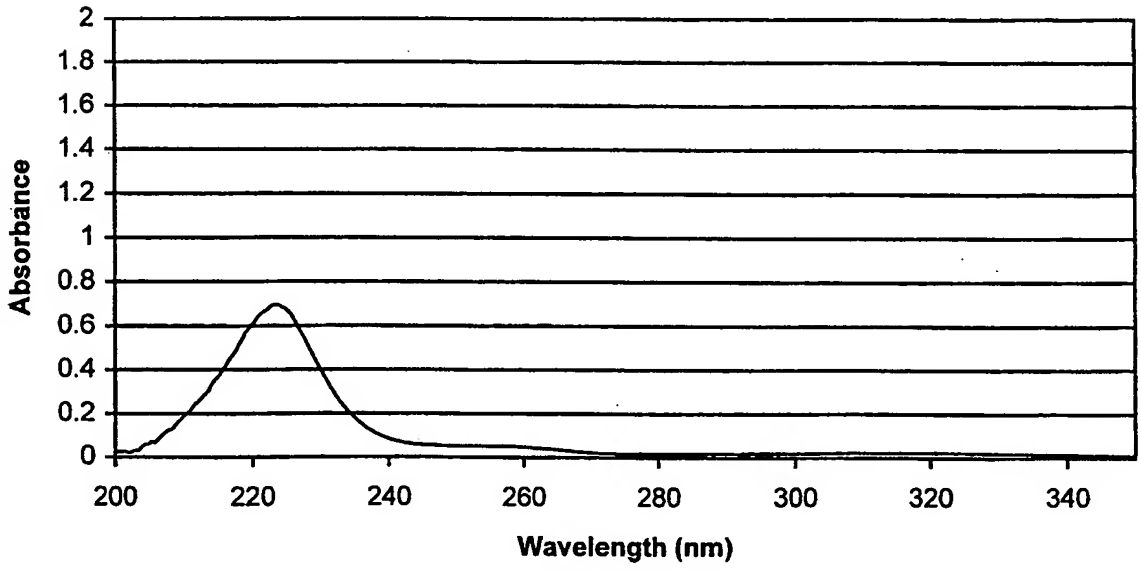


Figure 5B

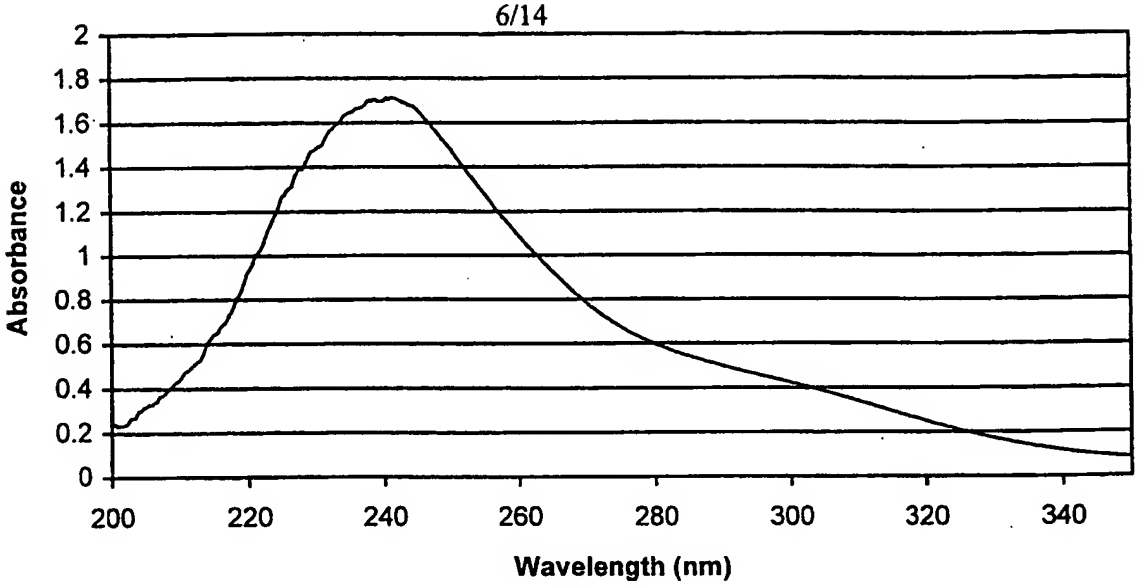


Figure 6A

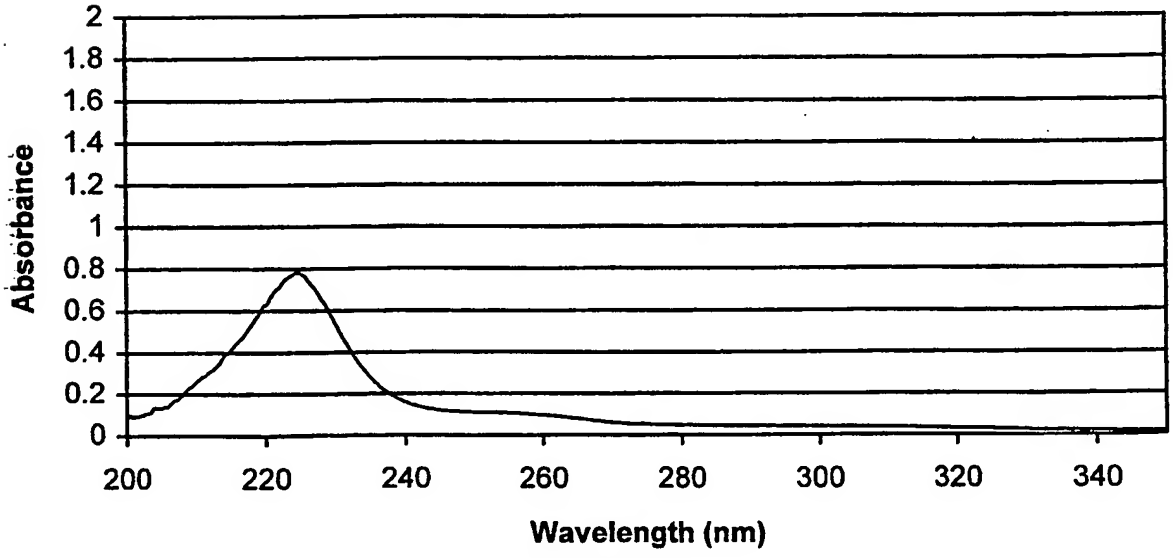
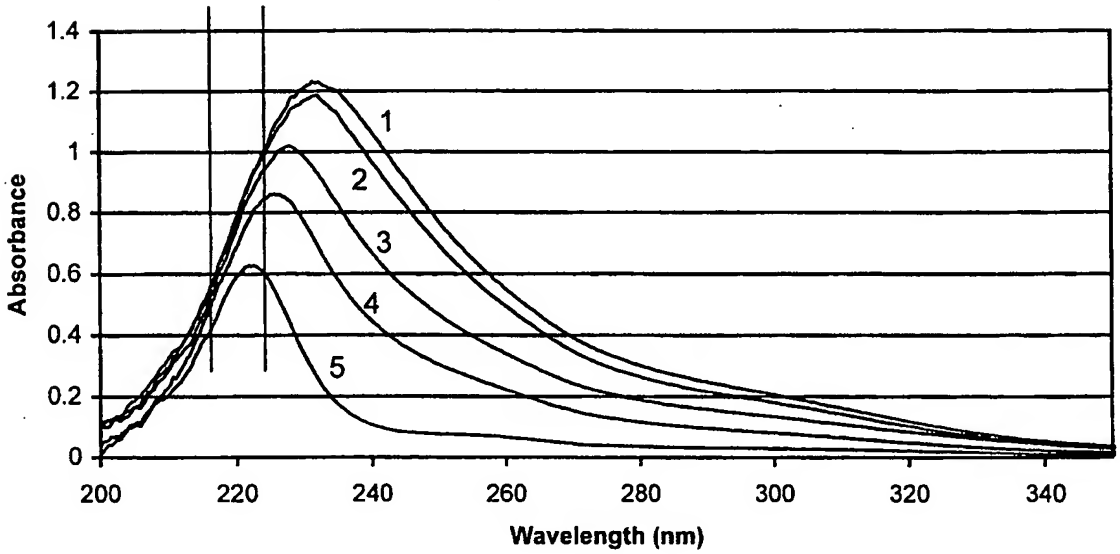


Figure 6B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Wavelength (nm)
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Figure 7

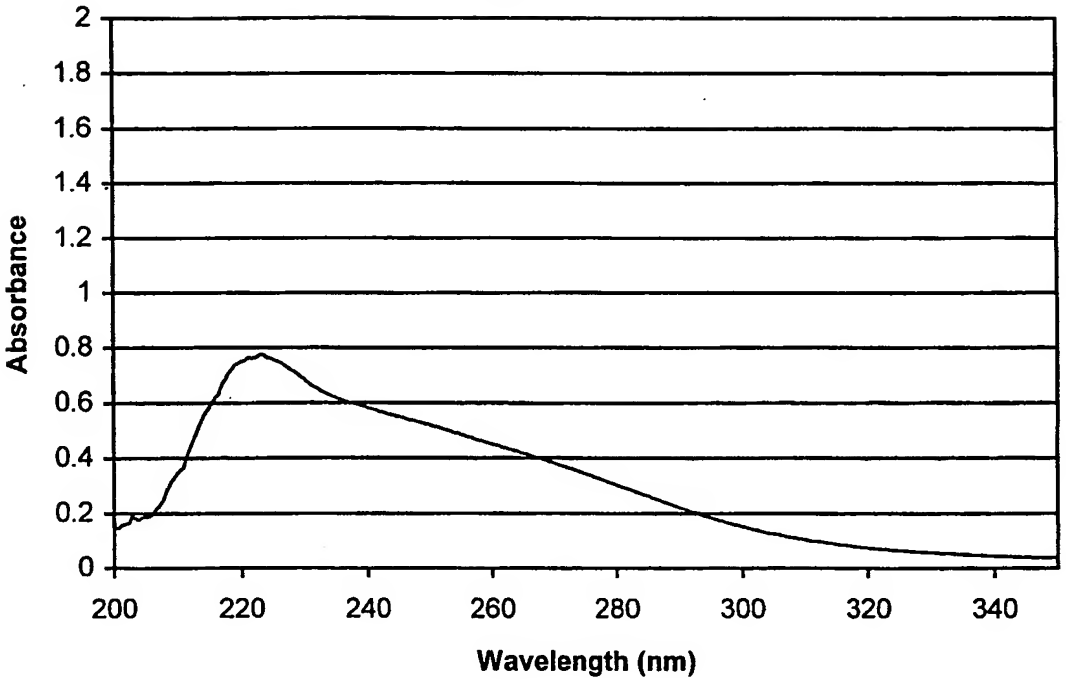


Figure 8A

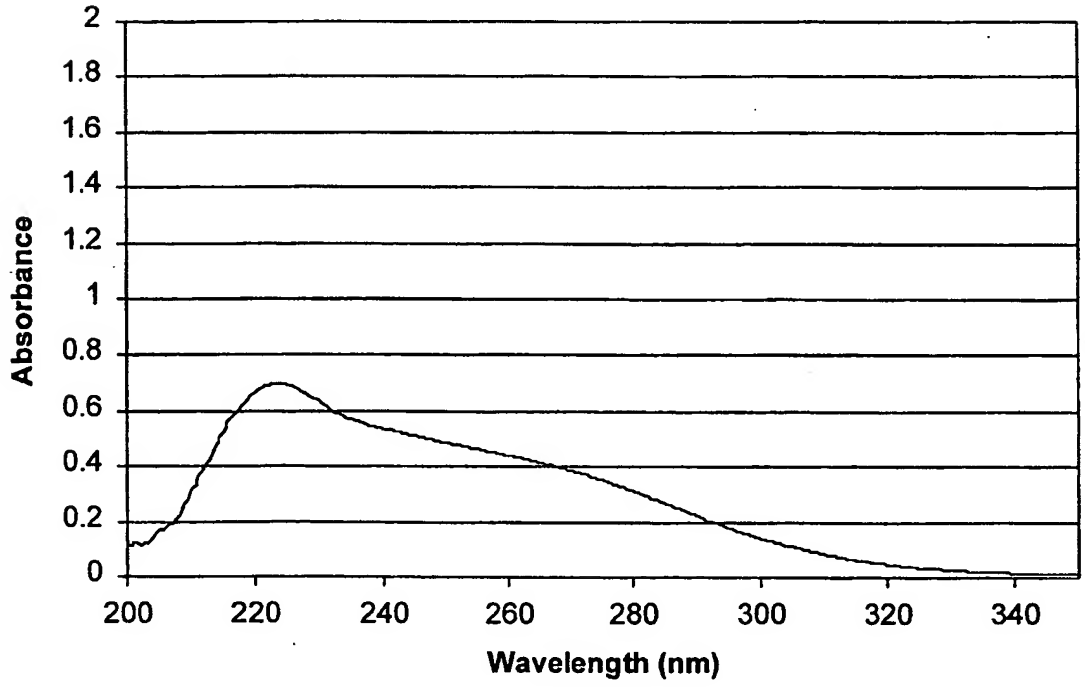
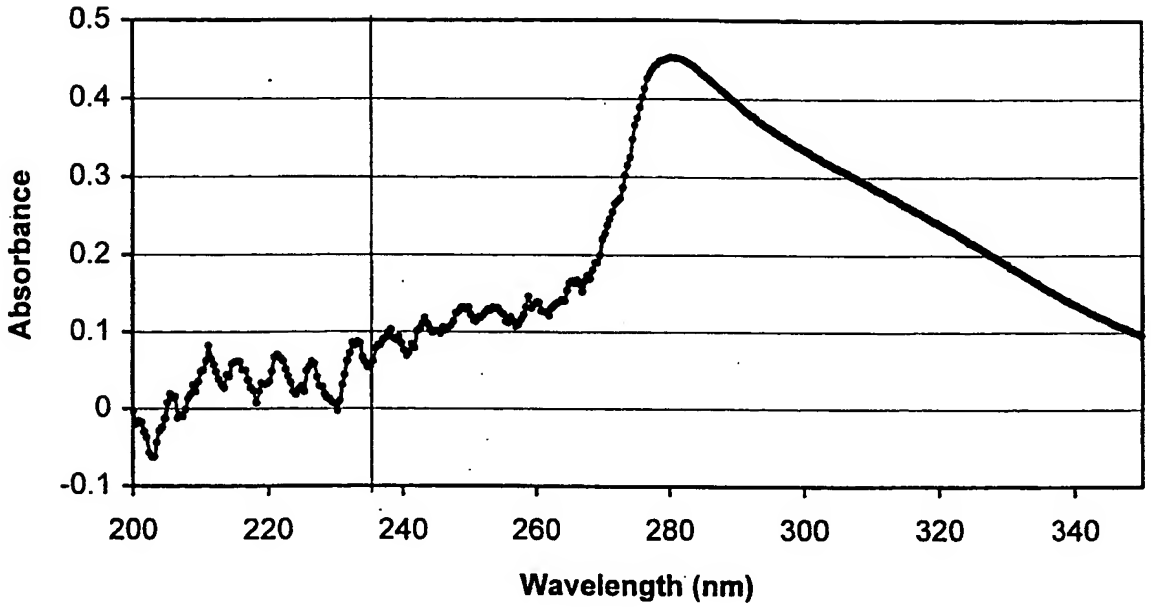


Figure 8B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Wavelength (nm)

Figure 9

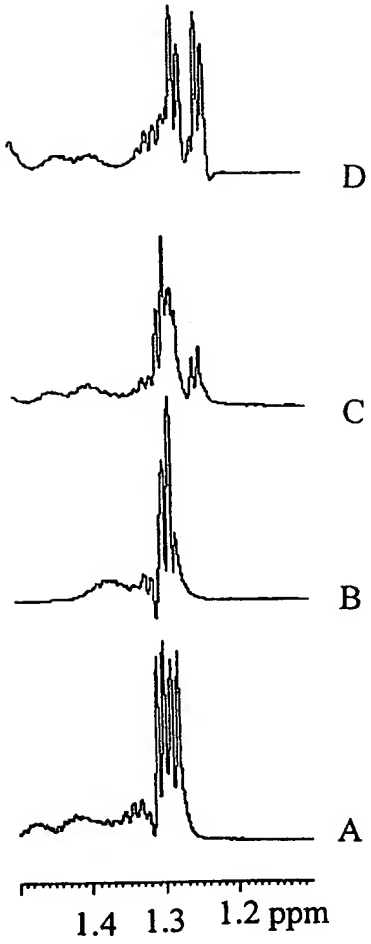

Figure 10

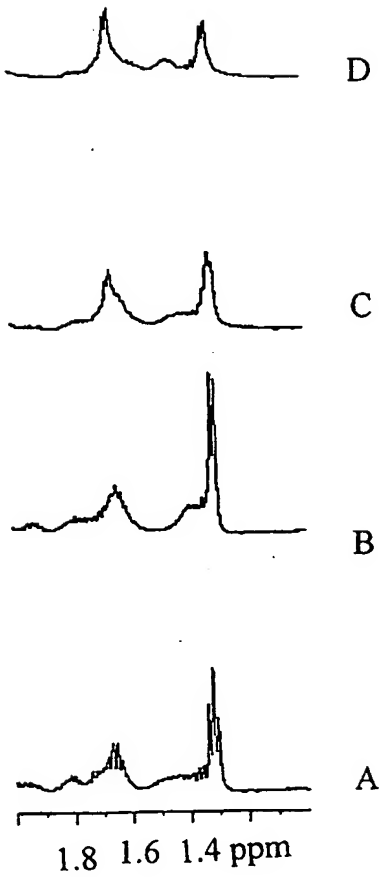

Figure 11

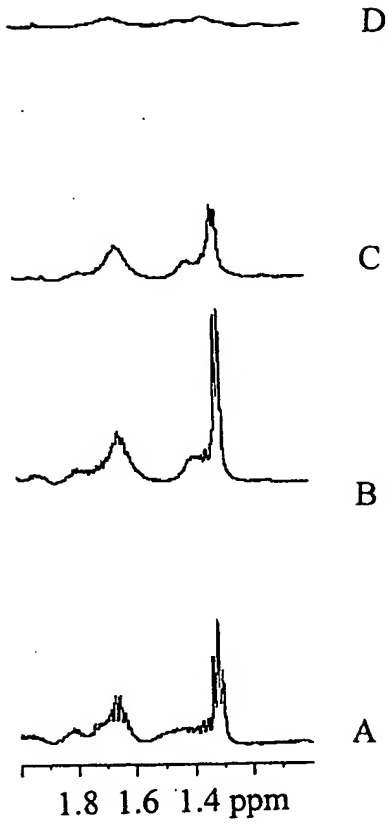

Figure 12

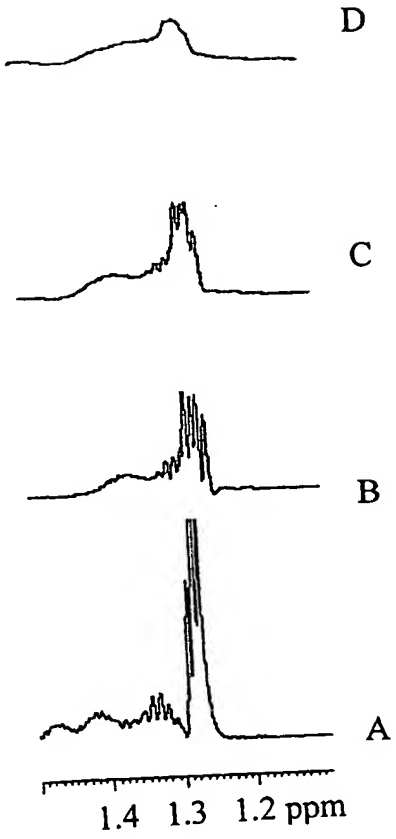

Figure 13

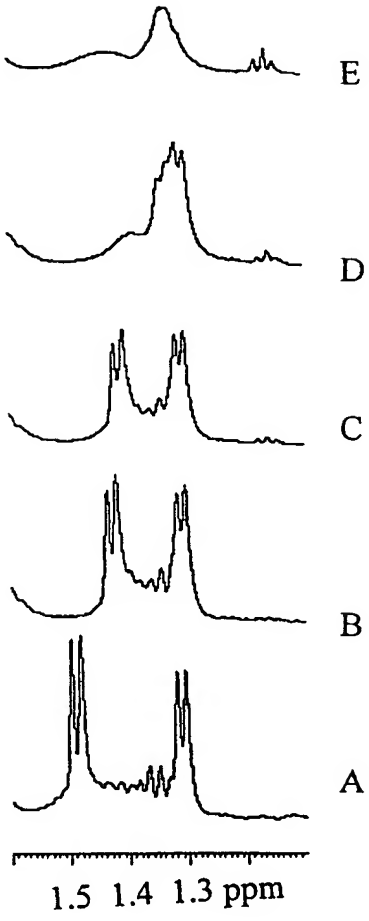

Figure 14

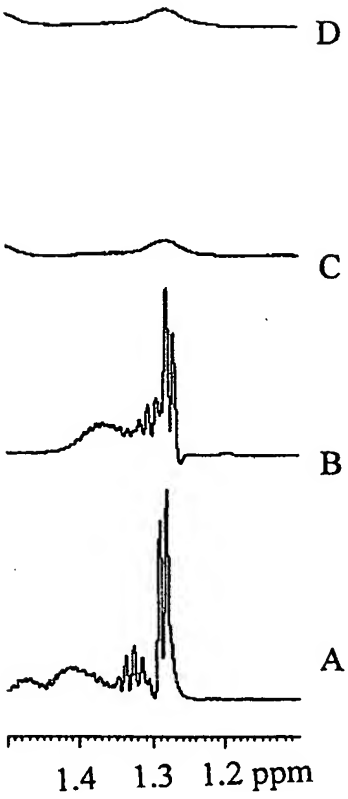

Figure 15

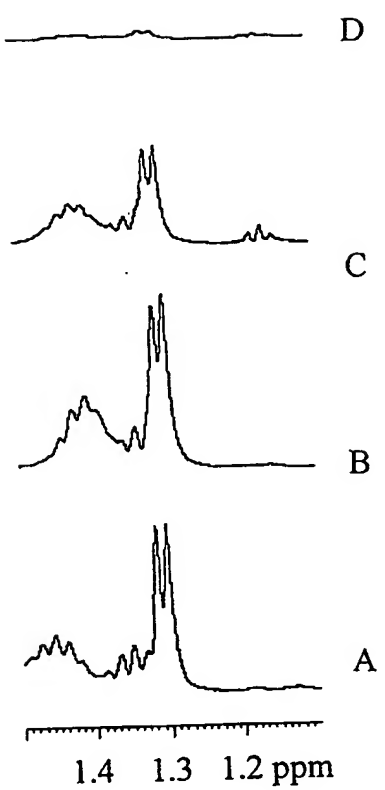

Figure 16

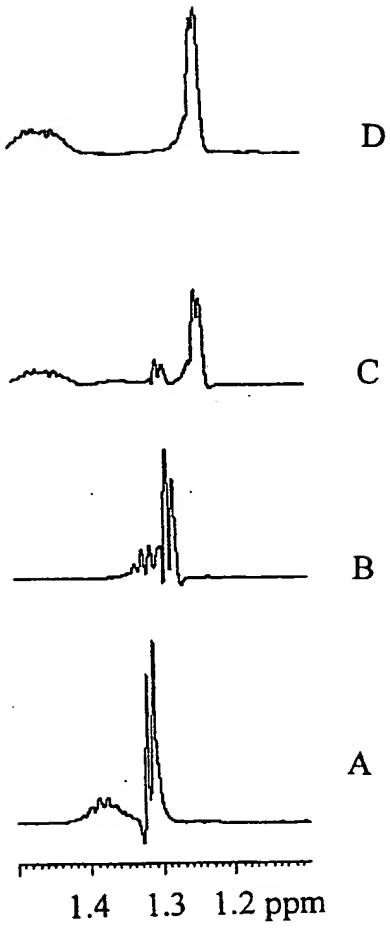

Figure 17

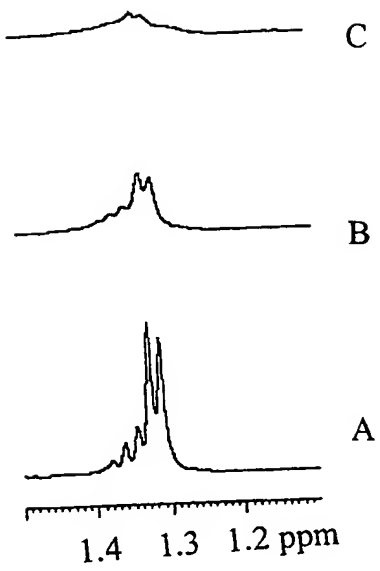

Figure 18

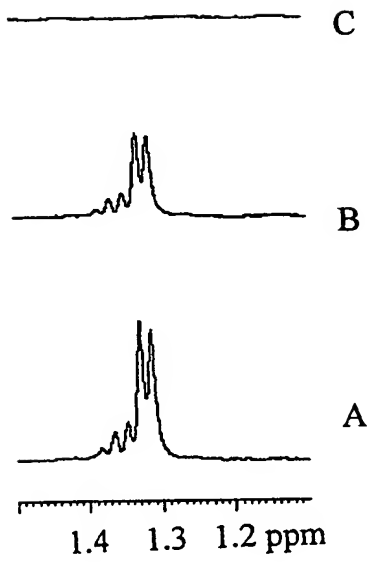

Figure 19

SEQUENCE LISTING

<110> Bar-Or M.D., David
Lau Ph.D., Edward
Winkler M.D., James V.

<120> Tests for the Rapid Evaluation of Ischemic States and Kits

<130> ISC007

<140> PCT/US99/_____
<141> 1999-10-01

<150> 60/115,392
<151> 1999-01-11

<150> 60/102,738
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Phe Ala Lys Thr Cys Val Ala Asp Glu Ser Ala Glu Asn Cys Asp Lys
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Lys Ala Glu Phe Ala Glu Val Ser Lys Leu Val Thr Asp Leu Thr Lys
225                 230                 235                 240

Val His Thr Glu Cys Cys His Gly Asp Leu Leu Glu Cys Ala Asp Asp
                245                 250                 255

Arg Ala Asp Leu Ala Lys Tyr Ile Cys Glu Asn Gln Asp Ser Ile Ser
            260                 265                 270

Ser Lys Leu Lys Glu Cys Cys Glu Lys Pro Leu Leu Glu Lys Ser His
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Cys Ile Ala Glu Val Glu Asn Asp Glu Met Pro Ala Asp Leu Pro Ser
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Cys Tyr Ala Lys Val Phe Asp Glu Phe Lys Pro Leu Val Glu Glu Pro
    370                 375                 380

Gln Asn Leu Ile Lys Gln Asn Cys Glu Leu Phe Glu Gln Leu Gly Glu
385                 390                 395                 400

Tyr Lys Phe Gln Asn Ala Leu Leu Val Arg Tyr Thr Lys Lys Val Pro
                405                 410                 415

Gln Val Ser Thr Pro Thr Leu Val Glu Val Ser Arg Asn Leu Gly Lys
            420                 425                 430
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Val Gly Ser Lys Cys Cys Lys His Pro Glu Ala Lys Arg Met Pro Cys
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Glu Lys Thr Pro Val Ser Asp Arg Val Thr Lys Cys Cys Thr Glu Ser
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Leu Val Asn Arg Arg Pro Cys Phe Ser Ala Leu Glu Val Asp Glu Thr
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22905

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :G0IN 21/00, 21/29, 31/22, 33/543, 33/00, 33/53; C12Q 1/00
US CL :Please See Extra Sheet.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 436/86, 518, 903, 904; 435/4, 7.9, 810; 422/55.61, 82.05, 82.09

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

STN, BIOSIS, MEDLINE, USAPATFUL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,227,307 A (BAR-OR et al) 13 July 1993, see entire document.	1-18
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Y		19-35
Y	US 5,290,519 A (BAR-OR et al) 01 March 1994, see entire document.	36-42

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 DECEMBER 1999	Date of mailing of the international search report 07FEB 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer PADMA BASKAR Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22905

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-35 and 36-42

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/22905

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

436/86, 518, 903, 904; 435/4, 7.9, 810; 422/55.61, 82.05, 82.09

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claims 1-35 and 36-42 drawn to a method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes an immunoassay.

Group II, claims 43-46 and 47-51 drawn to another method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes metal affinity diagnostic kit.

Group III, claims 52-55 drawn to monoclonal antibody directed to an epitope at the N-terminus of SEQ.ID NO.1.

Group IV, claims 56 drawn to monoclonal antibody directed to an epitope at the N-terminus of SEQ.ID NO.2.

Group V, claims 57-64 drawn to a calibrator composition and a method of calibrating an analyzer that detects or measures an ischemic event.

The inventions listed as Groups I to V do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I drawn to a method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes an immunoassay whereas Group II, drawn to another method for detecting the occurrence or non-occurrence of ischemic event and a kit which utilizes metal affinity diagnostic kit. These two methods are distinct and different in utilizing different steps, reagents and result in different outcome. Group III is a monoclonal antibody directed to an epitope at the N-terminus of the albumin which lacks the four, three, and two amino acid of SEQ. ID. NO. 1 where as Group IV, drawn to a structurally different monoclonal antibody directed to an epitope at the N-terminus of SEQ. ID. NO. 2. Group V to a calibrator composition and a method of calibrating an analyzer that detects or measures an ischemic event. This method is distinct and different from I and II in utilizing different steps, reagents and result in different outcome.

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(22) International Filing Date: 13 March 1998 (13.03.98)

(30) Priority Data:

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60/061,402	30 September 1997 (30.09.97)	US
09/037,933	10 March 1998 (10.03.98)	US

(71) Applicant *(for all designated States except US)*: NEUROMARK [US/US]; P.O. Box BA, Los Altos, CA 94023 (US).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)*: HARRINGTON, Michael, G. [US/US]; 4548 Leland Place, La Canada, CA 91011 (US).

(74) Agents: PASTERNAK, Dahna, S. et al.; Morrison & Foerster LLP, 755 Page Mill Road, Palo Alto, CA 94304–1018 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published

With international search report.

Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: DIAGNOSING NEUROLOGIC DISORDERS

(57) Abstract

Methods are described for identifying protein profiles which are specific for neurologic disorders. Methods of diagnosing neurologic disorders by detecting the levels of individual proteins in the disease–specific protein profile are also provided. Disease–specific protein profiles for Alzheimer's Disease, Parkinson's Disease, Multiple Sclerosis and schizophrenia are described.

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DIAGNOSING NEUROLOGIC DISORDERS

CROSS-REFERENCE TO RELATED APPLICATIONS

This invention claims priority to United States Provisional Patent Application Serial No. 60/036,586 filed March 14, 1997 and United States Provisional Patent Application Serial No. 60/061,402 filed September 30, 1997.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

This invention was made in part during work supported by a grant from the USPHS # NS30531. The government has certain rights in this invention.

TECHNICAL FIELD

This invention relates to diagnosis of neurologic disorders. More specifically, the invention provides methods of obtaining a disease-specific protein profile by determining proteins from a biological sample which are altered relative to normal controls. The invention also relates to methods of diagnosing neurologic disorders by determining the levels of the proteins which make up the disease-specific protein profile. The invention provides methods of diagnosing schizophrenia, Alzheimer's disease, Parkinson's disease, multiple sclerosis, subacute sclerosing panencephalitis (SSPE) and other neurologic disorders using disease-specific protein profiles.

BACKGROUND

Neurologic disorders such as schizophrenia, manic depression (bipolar disorder), Alzheimer's Disease, Parkinson's disease, multiple sclerosis, Huntington's disease and bacterial meningitis, are major public health concerns. Alzheimer's Disease (AD), for example, affects over 4 million Americans while schizophrenia affects approximately 2 million Americans. Alzheimer's disease has a gradual onset, usually beginning later in life. Symptoms of AD include difficulty with memory and loss of intellectual abilities severe enough to interfere with routine work or social activities. Confusion, language problems (such as trouble finding words), poor or decreased judgment, disorientation in place and time and changes in behavior or personality also occur. Eventually, AD leaves its victims

totally unable to care for themselves. Currently, the only way to confirm a diagnosis of AD is by post-mortem autopsy.

Parkinson's Disease (PD) affects over 1 million people in the U.S., with 20 new cases of PD per 100,000 people per year. PD is a chronic disease in which certain dopamine-producing neurons are destroyed. There is no definitive pre-mortem diagnosis available for PD. A diagnosis is based on a neurological examination, the severity of the symptoms and brain scans (CT or MRI) to help rule out other neurological diseases. If the symptoms are significant, a trial test of anitparkinsonian drugs may be used to diagnose the disease. Primary symptoms include stiffness, tremor, slowness and poverty of movement, difficulty with balance and walking. Secondary symptoms are depression; sleep disturbances; weight loss; dementia; drooling; difficulty with speech, breathing or swallowing; constipation and stopped posture.

Schizophrenia usually develops between adolescence and age 30 and is characterized by positive symptoms (delusions or hallucinations), negative symptoms (blunted emotions and lack of interest), and disorganized symptoms (confused thinking and speech, or disorganized behavior and perception). Schizophrenia can generally be treated with antipsychotic drugs which control most of the clinical symptoms of the disease.

Early and accurate diagnosis of neurologic disorders is essential for preventing relapses, selecting and monitoring the appropriate treatment regime, and reducing the risk of substance abuse and suicide. However, there are currently few specific and accurate molecular markers which would allow for diagnosis of AD, PD or schizophrenia in a living subject. Currently, neurologic disorders are usually only tentatively diagnosed by pre-mortem clinical evaluation or, definitively, by post-mortem autopsy of the brain. However, clinical evaluation of neurologic disorders is complex as the physician must rule out other problems or disorders which exhibit like symptoms. For example, PD can be confused with multiple sclerosis, ALS or Huntington's chorea. In the case of schizophrenia, drug abuse, seizures, major depressive or manic episodes, autism, and other personality disorders may present with psychotic symptoms similar to those seen in schizophrenia. Brain tumors, stroke, Alzheimer's and Parkinson's diseases can all present with symptoms including loss of memory, personality changes and aphasia.

The search for protein markers which are specific for a neurological disorder has proven elusive. Using two-dimensional gel electrophoresis (2DE), Hsich *et al.* (1996) *NEJM* 335(13):924-929 report that transmissible spongiform encephalopathies can be detected by the presence or amount of the protein in cerebrospinal fluid. U.S. Patent No. 4,874,694 to Gandy et al. claims a method of diagnosing certain neurologic disorders by incubating cerebrospinal fluid from a subject with 32-P labeled adenosine triphosphate (ATP) and a protein kinase capable of transferring phosphate from the ATP. Gel electrophoresis is then performed on the proteins and the autoradiography obtained compared to autoradiographs of known pathologies.

There are also references that describe diagnosis of neurologic disorders using only one biochemical marker. Most references describe one specific marker which is used for multiple neurologic disorders and where the specificity is low. The present invention has high specificity, for instance greater than 98% in CJD.

U.S. Patent No. 5,006,462 to Gattaz et al. describes diagnosis of schizophrenia by measuring increased enzymatic activity of phospholipase A2 (PLA2) in plasma samples when compared to nonschizophrenic controls. However, this assay could differentiate only approximately 70% of schizophrenic patients from healthy controls.

WO 95/05604 discloses methods of diagnosing Alzheimer's disease by detecting the presence of proteins altered in the olfactory neuroblast lysates of AD patients. WO 95/05395 discloses a method of diagnosing AD by measuring elevated levels of alpha-1 anti-trypsin or fibrinogen. U.S. Patent No. 5,429,947 to Merril et al. describes a method for diagnosing schizophrenia and Alzheimer's Diseases (AD) by detecting elevated levels of specific haptoglobin proteins in the bodily fluids of patients. This method is not specific for either of the two disorders listed. U.S. Patent No. 5,234,814 describes a method for assisting the diagnosis of AD by detecting amyloid precursor related proteins (APP) using an antibody which is reactive with the C-terminal of human amyloid precursor protein. Japanese Patent Nos. 3211461 and 2543606 disclose diagnosis of multiple sclerosis by measuring the amount of human tumor necrosis factor-alpha (h-TNF) in cerebrospinal fluid.

In diseases of the nervous system, the blood-brain barrier prevents diffusion of proteins from the brain to the peripheral blood system. However, the cerebrospinal fluid (CSF) is in immediate contact with the nervous system and proteins from this fluid can be

used as the molecular measure of pathology and responses to therapy. High resolution, two dimensional (2DE) separation techniques have yielded a detailed protein map of more than 2,000 individual components from CSF samples. The first dimension of 2DE separates proteins on the basis of charge, while the second dimension separates based on mass.

The present invention provides a specific method of generating disease-specific molecular markers. The disease-specific markers are obtained by screening a large number of proteins from a biological sample, identifying the particular proteins which are altered in subjects with neurological diseases by comparing their levels to levels found in normal subjects. Individually, these proteins do not distinguish the disorders from normal or other disease controls, but in combination, these protein profiles are very accurate at differentiating specific disorders. The particular proteins can then be isolated, purified, and used to generate antibodies. The antibodies are then used, for example, in Western blots or other immunoassays to diagnosis a specific disease condition in a sample. The disease-specific protein profiles are also called "bar-codes" in reference to the unique marking system used for merchandise. As with merchandise, the profiles expressed as bar codes provide a unique marking system for neurologic disorders. The bar-coding strategy of this invention allow for easy, rapid and specific assay for neurologic disease including schizophrenia, manic depressive disorder, Alzheimer's disease (AD), Parkinson's disease (PD), multiple sclerosis (MS) and Creutzfeldt-Jakob disease (CJD). It was the surprising discovery of the inventor that when the levels of the protein profile obtained from a patient is compared to normal controls, neurologic disorders can be diagnosed.

SUMMARY OF THE INVENTION

The present invention provides a method of diagnosing a neurological disorder in a biological sample, the method comprising: (a) obtaining a disease-specific protein profile by detecting proteins whose levels are altered in a subject having a neurological disorder when compared to levels of a healthy control; (b) obtaining the biological sample; (c) measuring the levels of the disease-specific protein profile obtained in step (a) in the biological sample; and (d) comparing the levels of the disease-specific protein profile in the biological sample to levels found in the healthy control; wherein altered levels of the

protein profile of the biological sample as compared to the healthy control is diagnostic of the neurological disorder.

In a preferred embodiment, the biological sample is cerebrospinal fluid. In another preferred embodiment, the neurologic disorder is selected from the group consisting of Alzheimer's Disease (AD), schizophrenia, Parkinson's disease (PD), multiple sclerosis, subacute sclerosing panencephalitis (SSPE) and transmissible spongiform encephalopathies. In a particularly preferred embodiment, the neurologic disorder is AD and the protein profile comprises proteins designated AD-1 through AD-18. In another particularly preferred embodiment, the neurologic disorder is PD and the protein profile comprises CIT-A, CIT-B, CIT-C (protein 129), CIT-1 (alpha 1 microglobulin subunit), CIT-2 (PGDS isoform), CIT-3 (PGDS isoform), CIT-4 (complement 4 gamma - C4γ) and CIT-5 (Apo-A1 lipoprotein). In yet another particularly preferred embodiment, the neurologic disorder is schizophrenia and the protein profile comprises protein 91, protein 48, protein 46, orosomucoid, prostaglandin D synthetase (PGDS), antithrombin III and fibrinogen degradation products. In other particularly preferred embodiments, the neurological disorders are multiple sclerosis, subacute sclerosing panencephalitis (SSPE), or Creutzfeldt-Jakob disease (CJD).

In another preferred embodiment, the disease-specific protein profile is obtained using 2 dimensional gel electrophoresis (2DE), Western blotting or immunoassay. In another preferred embodiment, the protein profile is measured by visualizing a 2DE gel, Western blotting or by immunoassay.

As will become apparent, preferred features and characteristics of one aspect of the invention are applicable to any other aspect of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a color reproduction of a 2DE gel electrophoresis of CSF fluid obtained from normal controls. Five positional standards, or landmarks, are identified in green. The position of five spots which are qualitatively or quantitatively altered in CSF from patients with disease are shown in red and labeled CIT-1 through CIT-5 in blue.

Figure 2 is a color reproduction of a 2DE gel electrophoresis of CSF fluid obtained from Parkinson's disease patients. Each of the protein spots comprising a PD-specific protein profile are marked in red.

Figure 3 is a color reproduction of a 2DE gel electrophoresis of CSF fluid obtained from Alzheimer's Disease patients. Eighteen spots comprising a AD-specific protein profile are marked in red.

Figure 4, panels A through C, are plots depicting the relative quantity of CIT-5 (Apo-A1 lipoprotein) in various Parkinson's patients. Each bar is representative of one patient. Panel A shows CIT-5 (Apo-A1 lipoprotein) plotted against the "H + Y" rating, a standardized index of disease severity. As disease severity increases, the amount of CIT-5 (Apo-A1 lipoprotein) decreases. On the x-axis, a "3" denotes long duration PD with dyskinesia, and moderate to high dopaminergic therapy, a "2" denotes medium duration, dyskinesia and low dopaminergic therapy and a "1" denotes short duration disease, no dyskinesia and no or minimum dopaminergic therapy. Panel B shows CIT-5 (Apo-A1 lipoprotein) plotted against the age of patient/duration of clinical disease. On the x-axis, "OL" refers to patients in whom the clinical disease onset occurred when they were older (above 50) with a long duration of disease, "YL" refers to a young-onset patients (below 50) with a long duration of disease, "OS" refers to old-onset and short duration disease, "YS" refers to young-onset and short duration. Panel C shows CIT-5 (Apo-A1 lipoprotein) plotted versus clinical parameters. The numbers on the x-axis are the same classifications as for Panel A. All panels in Figure 4 show that as PD severity or duration increases, the amount of CIT-5 (Apo-A1 lipoprotein) decreases.

Figure 5 is a graphical representation of a multiple discriminant function analysis plotting canonical discriminant functions obtained from the protein profiles of six PD patients (c); six AD patients (a) and two controls (b).

Figure 6 is a graphical representation of a multiple discriminant function analysis plotting canonical functions obtained from the protein profiles of six PD patients (c); four AD patients (b) and six controls (a).

Figure 7 shows the effect of various schizophrenia drug therapies on the level of protein 91. In normal controls, protein 91 has a normalized protein quantity (density units) of approximately 8. Individuals effected with schizophrenia have only around 4 units of the same protein. Patients on any of the three therapy regimes tested have less protein 91 than untreated or normal individuals.

Figure 8 shows the effect of various schizophrenia drug therapies on the level of protein 48. In normal controls, protein 48 has a normalized protein quantity of

approximately 7. Individuals affected with schizophrenia have around 13 units of the same protein. Patients on any of the three therapy regimes tested show a protein 48 level which is less than untreated individuals, but closer to the normal levels.

Figure 9 shows the effect of various schizophrenia drug therapies on the level of one isoform of prostaglandin D synthetase (PGDS). In normal controls, this PGDS isoform has a normalized protein quantity of approximately 8. Patients affected with schizophrenia have less than 4 units of the same protein. Samples obtained from patients on any one of the three therapy regimes show that PGDS levels remain lower than normal levels.

Figure 10 shows the effect of various schizophrenia drug therapies on the level of protein 46. In normal controls, protein 46 has a normalized protein quantity of approximately 8. Individuals affected with schizophrenia have around 13 units of the same protein. Patients on any of the three therapy regimes tested show a protein 46 level which were intermediate between untreated and normal levels.

Figure 11 depicts a two dimensional graph in which samples have been positioned based on their protein profile. A diagnosis of SSPE was made in sample labeled "E". This graph shows complete resolution of samples diagnosed as characteristic of SSPE from other samples, including multiple sclerosis patients.

MODES FOR CARRYING OUT THE INVENTION

Throughout this application, various publications, patents, and published patent applications are referred to by an identifying citation. The disclosure of the publications, patents, and published patent specifications referenced in this application are hereby incorporated by reference into the present disclosure to more fully describe the state of the art to which this invention pertains.

As used herein, certain terms will have specific meanings.

The term "neurologic disorder" is intended to refer the diverse group of disorders which affect the central or peripheral nervous system. The disorders may be acute or chronic and include, but are not limited to, schizophrenia, manic depression, Alzheimer's disease, Parkinson's disease, multiple sclerosis, transmissible spongiform encephalopathies (Creutzfeldt-Jakob disease in humans, mad cow disease in bovines and scrapie in sheep),

stroke, herpes simplex encephalitis, subacute sclerosing panencephalitis (SSPE), and brain damage associated with cerebral infarction.

As used herein, the terms "Alzheimer's Disease" and "AD" are used interchangeably to include the mental disorders characterized by memory loss, disorientation, confusion and the like. AD is also characterized by amyloid plaques and neurofibrillary tangles in the brain, which can be seen upon autopsy.

The terms "Parkinson's Disease" or "PD" are used interchangeably to include the neurologic disease characterized by tremor, rigidity, akinesia (freezing), postural disturbance or balance problems. Fatigue, depression, anxiety, restlessness, poor handwriting, quieting of voice, change in posture, muscle aches, cramping or paresthesias (tingling feelings) are also reported. Diagnosis of PD can be complicated because of "Parkinsonism look-alikes" or conditions which share clinical features with PD. PD can be divided into different subtypes based, for example, on age of onset, duration or amount of dyskinesia.

The term "schizophrenia" is intended to include the group of mental disorders characterized by disruptions in thinking and perception. In a clinical evaluation, schizophrenia is commonly marked by auditory hallucinations (especially hearing voices), disorganized thought processes, delusions, and the absence of emotion or affect. Clinical diagnosis of schizophrenia is complex, and as with most psychiatric diagnostic categories, is effected at the syndromal level. (see, Michael Flaum, (1995) "The diagnosis of schizophrenia" in CONTEMPORARY ISSUES IN THE TREATMENT OF SCHIZOPHRENIA, Shriqui and Nasrallah eds., American Psychiatric Press, Inc, Washington D.C.).

The disorder known as schizophrenia can be divided into several subtypes or categories. The term "schizoaffective" is intended to mean a category of schizophrenia patients who also exhibit mood disorders, for example, bipolar disorder. Schizoaffective disorder is generally characterized by common schizophrenia symptoms in combination with major depressive or manic episodes. "Post partum" schizophrenia refers to a the category of schizophrenia which exhibits common symptoms but onsets in women after giving birth. The term "schizophrenic" refers to a person who suffers from schizophrenia.

The term "transmissible spongiform encephalopathies (TSEs)" is intended to refer to a group of neurodegenerative diseases. In humans, these diseases include Creutzfeldt-Jakob disease (CJD), Gerstmann-Staussler-Scheinker syndrome, Fatal Familial Insomnia,

and Kuru. (*see, e.g.*, Brown *et al.* in NEURODEGENERATIVE DISEASES, Calne *ed.*, W.B. Saunders, Philadelphia (1994); Medori *et al.* (1992) *N. Engl. J. Med.* 326:444-449). In animals, the TSEs include sheep scrapie, bovine spongiform encephalopathy, transmissible mink encephalopathy, and chronic wasting disease of captive mule deer and elk (Gajdusek (1990) *Subacute Spongiform Encephalopathies: Transmissible Cerebral Amyloidoses Caused by Unconventional Viruses.* pp. 2289-2324 In: *Virology*, Fields, ed. New York: Raven Press, Ltd.). All TSEs are characterized by the same hallmarks: a spongiform degeneration, reactive gliosis in the cortical and subcortical gray matters of the brain, and transmission when experimentally inoculated into laboratory animals including primates, rodents and transgenic mice.

A biological "sample" as used herein includes a variety of sample types obtained from an individual and is typically used in a diagnostic procedure or assay. The definition encompasses blood, cerebrospinal fluid (CSF) and other liquid samples of biological origin, solid tissue samples such as biopsy specimen or tissue cultures or cells derived therefrom and the progeny thereof. Methods of collecting and storing tissue or fluid samples are known to those of skill in the art. The definition also includes samples that have been manipulated in any way after their procurement, such as by treatment with reagents, solubilization or enrichment for certain components, such as proteins or polynucleotides. The term "biological sample" encompasses a clinical sample, and also includes cells in culture, cell supernatants, cell lysates, serum, plasma, biological fluid and tissue samples.

The terms "level" or "levels" are used to refer to the presence and/or amount of protein, and can be determined qualitatively or quantitatively. A "qualitative" change in the protein level refers to the appearance or disappearance of a protein spot that is not detectable or is present in samples obtained from normal controls. A "quantitative" change in the levels one or more proteins of the profile refers to a measurable increase or decrease in the protein levels when compared to a healthy control.

The term "protein profile" refers to a pattern of specific proteins whose levels are altered in samples obtained from subjects with neurologic disorders when compared to a healthy control. The profile may be expressed in many forms, including, for example, as a "molecular bar code" composed of the proteins of the profile. A disease-specific protein profile is obtained by comparing the level of a variety of proteins in a sample taken from a

healthy control to the levels found in samples taken from affected individuals. Individually, the proteins which are altered in the affected individuals may not be diagnostic of the disorder, but, when viewed together as a profile, are disease-specific. The proteins which comprise the profile may all be increased, decreased, present or absent with respect to healthy levels. More often, some of the proteins will be increased and some will be decreased as compared to the control.

The proteins which make up the disease-specific protein profile may be identified by their common name, their structure, their sequence, by the antibodies which recognize them or by their migration pattern in gel electrophoreses. One or two dimensional gels may be used. Methods of 2DE are known in the art, and described herein in Example 1. For example, a protein profile for AD comprising 18 proteins has been identified. Two of the proteins have been identified by their common names, Haptoglobin 1 precursor subunits, while sixteen are identified by their migration pattern in gel electrophoresis. A protein profile for PD comprises 9 proteins, including CIT-A, CIT-B, protein 129, alpha 1 microglobulin subunits, two PGDS isoforms, complement 4 gamma and Apo-A1 lipoprotein. The profile for schizophrenia described in the present application includes proteins 48, 46 and 91. To date, the amino acid sequence of these proteins is not known. Accordingly, these proteins are identified herein by randomly-assigned numbers corresponding to the location on a 2DE gel where the protein appears under the conditions described herein. Proteins 5, 48, 46 and 91 have been previously identified in Harrington et al. (1985) *Clinical Chemistry* 31(5):724. As shown in Figure 1 of this reference, these proteins migrate in response to electrophoresis in a characteristic manner.

The proteins of the protein profile may also be identified, for example, by their isoelectric focusing point (pI) and molecular weight (MW) in kilodaltons (kD). For example, some of the proteins identified herein have the following pI and apparent MW:

Table 1

Protein Number (Name)	pI	MW (kD)
protein 48	6.2	46
protein 46	6.0	48
protein 91	5.7	80
108	5.3	30
110	5.1	32
protein 2 (PGDS)	5.5	20
protein 5 (PGDS)	5.7	20
protein 129	6.1	25
CIT-A	5.2	94
CIT-B	5.58	54
CIT-C	5.54	26
CIT-1 (alpha 1 microglobulin subunit)	5.12	30.851
CIT-2 (PGDS isoform)	5.2	25
CIT-3 (PGDS isoform)	5.2	25
CIT-4 (complement 4 gamma)	6.37	33.074
CIT-5 (Apo-A1 lipoprotein)	5.46	23.320
AD-1	6.37	150
AD-2	5.46	65
AD-3	6	63
AD-4	6.09	63
AD-5	6.09	63
AD-6	64	6.6
AD-7	64	6.6
AD-8	62	6.9
AD-9	62	7
AD-10	51	5.46
AD-11 (Haptoglobin 1 precursor subunit)	42	5.47
AD-12 (Haptoglobin 1 precursor subunit)	42	5.47
AD-13	30.5	5.48
AD-14	30.5	5.48
AD-15	30	5.1
AD-16	30	5.1
AD-17	28	5.18
AD-18	17	6.68

The pI and molecular weight values are intended to include a range which includes any variations which may occur due to experimental conditions as would be known by one of skill in the art. In the neutral pH region, pIs will generally range up or down 0.5 from the value given. At higher pHs, the range may be $\pm$ 1. Molecular weight ranges will usually be within 5 kilodaltons of the value given, although glycoproteins may migrate

anomalously in SDS PAGE gels and give show MW values which are higher than the actual MW.

The proteins which comprise the disease-specific profiles can be quantified, for example, by measuring the units of protein concentration or normalized density units.

Alternatively, the protein profile can be visualized, for example, from electrophoresis, in particular, 2DE. Electrophoretic methods are well-known to those skilled in the art and are described in detail herein. Visualizing proteins may be performed by any method known in the art. Coomassie blue and silver stain, for instance, are well known methods of visualizing proteins on gels. Western blotting and autoradiography of gels can also used to identify the disease-specific protein profiles.

It is intended that the protein profiles used in the methods described herein be composed of at least 2 different proteins, or alternatively, at least two isoforms or glycoforms of the same protein. Preferably the protein profiles are comprised of between 3 and 20 proteins or glycoforms, more preferably, between 4 and 15, and even more preferably 5 to 10. Glycoforms of the same protein can be distinguished by methods known in the art. As described above, glycoforms of proteins are known to migrate differently in 2DE, as determined by molecular weight and isoelectric focusing points. Therefore, a protein profile could be prepared by measuring or visualizing glycoforms of the same protein. Although the only limit on the number of proteins which can make up a profile is the number of proteins in the sample, for ease of analysis, less than 20 is preferred.

In addition to differentiating between neurologic disorders, it is also intended the protein profiles can be used to differentiate between diagnostic categories of individual diseases. In schizophrenia, for example, post-partum schizophrenia or schizoaffective disorder may produce protein profiles which are distinct from conventional schizophrenia. Similarly, early-onset Alzheimer's disease may present with a different protein profile from the more common age-associated AD. Moreover, it is contemplated that an individual subject may have a unique protein profile, for instance, because they are suffering from more than one neurologic disorder. In addition, the protein profile can be used to distinguish specific cell damage or brain region damage based on the appearance of proteins unique to their origin.

A "healthy control" or "normal control" is a biological sample taken from an individual who does not suffer from a neurologic disorder. A "negative control," is a sample that lacks any of the specific analyte the assay is designed to detect and thus provides a reference baseline for the assay.

As used herein, the term "cerebrospinal fluid" or "CSF" is intended to include whole cerebrospinal fluid or derivatives or fractions thereof well known to those of skill in the art. Thus, a cerebrospinal fluid sample can include various fractionated forms of cerebrospinal fluid or can include various diluents added to facilitate storage or processing in a particular assay. Such diluents are well known to those of skill in the art and include various buffers, preservatives and the like.

As used herein, the term "2-dimensional gel electrophoresis" or "2-D gel electrophoresis" or "2DE" is intended to refer to the two dimensional migration of proteins in solution or suspension in the presence of an electrical field. Methods of 2DE are well known to those of skill in the art. See generally, R. Scopes (1982) *Protein Purification,* Springer-Verlag, N.Y. and Deutscher (1990) *Methods in Enzymology Vol. 182: Guide to Protein Purification,* Academic Press Inc., N.Y.). For example, 2DE relies on isoelectric focusing (IEF) in either carrier ampholyte gradient gels or immobilized pH gradients for one dimension and SDS-polyacrylamide gels for the second dimension. The amount of polyacrylamide to be used in making the gels can be readily determined by a skilled artisan.

As used herein, an "immunoassay" is an assay that utilizes an antibody to specifically bind to the antigen. The immunoassay is thus characterized by detection of specific binding of the proteins of the protein profile to antibodies as opposed to the use of other physical or chemical properties to isolate, target, and quantify the analyte. Direct immunoassay and competitive immunoassays are described in detail below.

As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of immunoglobulin genes. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

The basic immunoglobulin (antibody) structural unit is known to comprise a tetramer or dimer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50–70 kD). The N–terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these variable regions of the light and heavy chains respectively.

Antibodies may exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to V_H–C_H1 by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the $F(ab)'_2$ dimer into an Fab' monomer. The Fab' monomer is essentially an Fab with part of the hinge region (*See, Fundamental Immunology,* W.E. Paul, ed., Raven Press, N.Y. (1993) for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies.

The phrase "specifically binds to a protein" or "specifically immunoreactive with," when referring to an antibody refers to a binding reaction which is determinative of the presence of the protein in the presence of a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies preferentially bind to a particular protein and relative binding to other proteins does not occur in significant amounts. Specific binding to a protein under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, antibodies can be raised to the protein markers specific for schizophrenia. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid–phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. *See* Harlow and

Lane (1988) *Antibodies. A Laboratory Manual,* Cold Spring Harbor Publications, New York, for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity.

As used herein, the term "subject" refers to a mammal and includes, but is not limited to, humans, bovine, mink, sheep, elk, cats and deer.

I. Obtaining Disease-Specific "Protein Profiles"

The present invention includes a method of obtaining a disease-specific protein profile which may be expressed as a molecular bar code. The familiar bar code system of grocery and other retail stores used to price goods reduces errors, saves time and helps keep inventory. In the disease context, unique bar codes will provide accurate diagnosis, patient management and therapeutic monitoring.

The protein profiles expressed as molecular bar codes are obtained from a biological sample taken from a subject known to have a particular disorder, and preferably a neurologic disorder. Sample collection is discussed in detail below. The proteins of the sample are then detected. Preferably, the sample is first subjected to two-dimensional gel electrophoresis (2DE) to distinguish individual proteins by their migration patterns. The 2DE gel is then visualized, preferably by silver staining and quantified, for example by image analysis. Altered levels of particular proteins in the sample are then obtained by comparing the protein levels in the sample to levels of a healthy control or a control known to have a disorder, including a neurologic disorder. Preferably, a computer algorithm and a database of protein levels from CSF is used to determine which particular proteins are increased or decreased in the sample. In this way, from the thousands of proteins present in biological samples, those which are altered in particular diseases can be identified. All or some of the specific proteins which are altered are called the protein profile.

When 2DE is performed, it is preferable to use at least two different stained electrophoresis gels to generate a protein profile. Protein spots which are present in every gel are designated as positional standards or "landmarks." These landmark spots are aligned as between at least two gels, and the rest of the spots digitized according to their relation to the landmarks. Subsequently, a computer generated algorithm or the like can be programmed to compare the test CSF with healthy controls and determine which spots are different in one gel from another, and which are similar.

The algorithm used to determine the protein profile is a multi-step process. Computer programs can used to compare and contrast intensity of spots on 2DE gels, for instance commercially available programs such as "Melony" (BioRad). Development of computer generated algorithms is within the skill of an ordinary programmer. Generally, the protein spots on the 2DE gel are first identified using any spot-finding method known in the art. Preferably, an edge detector or peak identifier is used. Various parameters are recorded and stored for each spot, including, for example, x and y co-ordinates corresponding to pH and molecular weight along with total integrated density. Next, all spots are compared from one sample to one another, using a master gel retaining all data. The protein spots are either matched or unmatched and noted by their location on the gel. The differences can be qualitative (e.g. unmatched spots) or quantitative (statistically significant variations of normalized protein quantity).

The compared gel data is then split into groups of each disease and normal controls. Similarities and differences are noted as described above. All of the different proteins are then further analyzed in subsets of patients within each disease compared to controls.

The above-described steps each use conventional statistics for judging differences in data. Preferably, means, standard deviations, a student T-test, and analysis with appropriate degrees of freedom are employed. Other methods known to those in the art may also be suitable. Any changes are verified from one population by testing in a second population and only those changes that are replicable are confirmed as valid.

Using the novel method described here, we can define a diagnostically powerful profile for each disease and even for disease sub-types. This is achieved simply when an individual protein is so discriminative, but this is rare and likely to be unusual. One known example is the 14-3-3 marker for CJD. More commonly, a number of protein spots are altered in a statistically significant manner between disease and controls, but with overlap that is not useful for any one individual protein, but when analyzed together possess powerful discrimination. This can be achieved in a number of different ways, but most appropriately using multi-dimensional discriminant function analysis. Preferably, the algorithm is standard statistical software from Unistat. Other statistical methods will be known to those skilled in the art. Either linear or nonlinear discriminant function analysis can be performed, and alternative approaches can include principal component analysis, factor analysis, or cluster analysis. All variable proteins were examined for combinations

that would provide the best discrimination and then the best discrimination is investigated for the least number of proteins. Once this is achieved (usually 10 or less proteins with most discrimination coming from the first 5 proteins) then these proteins were used to test a second, new population of samples. Only when these same proteins gave discriminating power in the second population was this group of proteins considered for the profile. Once so identified, the profile can be used to define a multidimensional space of discriminant function that allows subsequent samples to be placed with confidence in one group or another.

Thus, the present invention provides a method for identifying protein profiles which are specific for a disease or an individual. The diseases which can be diagnosed and monitored using the novel method described herein include, but are not limited to, schizophrenia, Alzheimer's disease, Parkinson's disease, multiple sclerosis, manic depression, Huntington's disease, viral encephalitis, stroke, dementia, headaches, bacterial meningitis, insomnia and transmissible spongiform encephalopathies.

A. Protein Profile for Alzheimer's Disease (AD)

In one embodiment, a protein profile specific for AD is identified. At least about 18 protein spots have been found to make up a protein profile. These spots, AD-1 through AD-18 are identified by their molecular weight and isoelectric point in Table 1. The AD profile identified herein is comprised of 18 qualitative markers. Another profile specific for AD has been identified using a combination of proteins that include 2 of those qualitative markers with 7 other proteins that are altered in various diseases.

B. Protein Profile for Parkinson's Disease (PD)

The present invention also includes a protein profile for PD. The present inventor has discovered at least 3 qualitative and 5 quantitative proteins making up a disease-specific protein profile. The 3 qualitative markers of the profile (*i.e.* spots that are not present in normal controls) are protein 129, CIT-A (molecular weight of approximately 94 kDa and isoelectric point of approximately 5.2), and CIT-B (molecular weight of approximately 54 kDa and isoelectric point of approximately 5.58). The quantitative markers, or spots which increase or decrease compared to normal are: Apo-A1 lipoprotein; alpha 1 microglobulin subunit; two PGDS isoforms and complement 4 gamma. All spots decrease in PD patients except one PGDS isoform (CIT-2) which is increased.

C. Protein Profile for Schizophrenia

In another embodiment, a protein profile specific for schizophrenia is identified. As described in the Examples, using the methods of the present invention a schizophrenia-specific profile made up of seven proteins was identified. These seven proteins, protein 91, protein 48, protein 46, orosomucoid, prostaglandin D synthetase (PGDS), antithrombin III and fibrinogen degradation products are altered in schizophrenic subjects when compared to normal controls. In particular, protein 91, orosomucoid (also known as α_1-acid glycoprotein), PGDS, and antithrombin III have a relative concentration which is decreased relative to normal. Proteins 48 and 46 are increased relative to normal. Fibrinogen degradation products are absent in normal controls and appear in schizophrenic samples.

D. Differentiation between Neurologic Disorders

Once a protein profile has been identified, this group of proteins can be used to define the multidimensional space of discriminant function that allows subsequent samples to be placed with confidence in one group or another. As shown in Figures 5, 6 and 11, multiple discriminant function analyses can be used to distinguish between various neurological conditions. Figure 5 shows that PD patients (c) and AD patients (a) can be distinguished from each other and from healthy controls (b) where the multidimensional discriminating space is plotted in 2-dimensions. Similarly, in Figure 6, PD patients (c) separate from AD (b) and from controls (a). Figure 11 illustrates the diagnosis of subacute sclerosing panencephalitis (SSPE) patients in Group 5, individually labeled E with complete resolution from the other patient groups.

In other embodiments, protein profiles specific for multiple sclerosis, Parkinson's disease, subacute sclerosing panencephalitis (SSPE) or Creutzfeldt-Jakob disease are disclosed. The ability to distinguish between SSPE and multiple sclerosis is an especially interesting and novel finding as conventional biochemical techniques cannot distinguish between these two disorders.

II. Diagnosis of Neurologic Disorders Using Disease-Specific Protein Profiles

The disease-specific protein profile obtained as described above can then be used for diagnosis of the specific disorder. The individual proteins of the protein profiles can be detected or quantified by any of a number of means well known to those of skill in the art. These may include visualization techniques, analytic biochemical methods such as electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), hyperdiffusion chromatography, partial amino acid sequence determination and the like, or various immunological methods such as fluid or gel precipitation reactions, immunodiffusion (single or double), immunoelectrophoresis, radioimmunoassay (RIA), enzyme–linked immunosorbent assays (ELISAs), immunofluorescent assays, western blotting, and the like.

In one aspect, a qualitative change in one or more proteins of the protein profile is determined. Qualitative changes include the appearance of a protein spot that is not detectable in samples obtained from normal controls or the disappearance of a protein spot which is detectable in normal controls but not in the sample taken from an affected subject.

In another aspect, a quantitative change in one or more proteins of the profile is measured. Preferably, the protein profile is quantified using 2DE, immunoassay or Western blotting. The concentration of protein levels may be expressed in absolute terms, for example, optical density as read by image analysis. Alternatively, the concentrations can be expressed as a fraction, relative to normal levels of the same protein.

The proteins which make up the protein profile can be individually isolated and, optionally purified. Methods of isolating and purifying proteins from 2DE gel are known in the art. The isolated proteins are then identified, for example, by partial amino acid sequence, mass spec or carbohydrate analysis. Monoclonal or polyclonal antibodies can then be generated to the isolated proteins by methods known in the art. *See* Harlow and Lane (1988) *Antibodies. A Laboratory Manual,* Cold Spring Harbor Publications, New York, for a description of methods of generating antibodies. Used in combination, these specific antibodies are used to diagnosis the specific disease in a subject by analyzing a biological sample from the subject.

The collection of biological sample and subsequent testing for a disease-specific protein profile is discussed in more detail below.

A) Sample Collection and Processing

The disease-specific protein profile is preferably quantified in a biological sample derived from a mammal, more preferably from a human patient. A biological sample is a sample of biological tissue or fluid that contains a protein profile concentration that may be correlated with protein profile levels of a healthy control. Particularly preferred biological samples include but are not limited to, plasma, urine, serum, neurological tissue and cerebrospinal fluid.

The biological sample may be pretreated as necessary by dilution in an appropriate buffer solution or concentrated, if desired. Any of a number of standard aqueous buffer solutions, employing one of a variety of buffers, such as phosphate, Tris, or the like, at physiological pH can be used.

In a preferred embodiment, assays are performed using cerebrospinal fluid (CSF). Obtaining and storing CSF are well known to those of skill in the art. Typically CSF is obtained by lumbar puncture. The CSF may be diluted by the addition of buffers or other reagents well known to those of skill in the art and may be stored for up to 24 hours at 2–8°C, or at –20°C or lower for longer periods, prior to measurement of the protein profile. In a particularly preferred embodiment, the CSF is stored at –70°C without preservative indefinitely.

B) Electrophoretic Assays

As indicated above, a protein profile specific for schizophrenia may be obtained form cerebrospinal fluid using electrophoretic methods. Means of detecting proteins using electrophoretic techniques are well known to those of skill in the art (see generally, R. Scopes (1982) *Protein Purification,* Springer–Verlag, N.Y.; Deutscher, (1990) *Methods in Enzymology Vol. 182: Guide to Protein Purification.,* Academic Press, Inc., N.Y.). In a preferred embodiment, the protein profile is obtained using two-dimensional electrophoresis. A particularly preferred separation relies on isoelectric focusing (IEF) in carrier ampholyte gradient gels or immobilized pH gradients for one dimension and 14% polyacrylamide gels for the second dimension. A detailed protocol for two-dimensional electrophoresis is provided in Examples below.

Proteins separated on 2DE gels can be visualized by any method known in the art. For example, Coomassie blue staining, gold staining, silver staining and the like may be

used to visualize proteins directly on the gels. (see, generally, *Current Protocols in Molecular Biology*, Ausubel *et al.* eds. (1996) § 10.6 to 10.8 and references therein). Alternatively, the proteins on the 2DE gel can be transferred or blotted onto a membrane and probed with an antibody. Methods of blotting and immunostaining are known to those of skill in the art. (see, e.g., Ausubel (1996), *supra*, § 10.8). In a preferred embodiment, the present invention obtains a protein profile by silver staining and, subsequently, by immunostaining.

C) Immunological Binding Assays

In another embodiment, the protein profile is obtained or measured using any of a number of well recognized immunological binding assays *(see, e.g.,* U.S. Patents 4,366,241; 4,376,110; 4,517,288; and 4,837,168). For a review of the general immunoassays, see also *Methods in Cell Biology Volume 37. Antibodies in Cell Biology,* Asai, ed. Academic Press, Inc. New York (1993); *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991).

Immunological binding assays (or immunoassays) typically utilize a "capture agent" to specifically bind to and often immobilize the analyte (in this case a protein of the specific protein profile). The capture agent is a moiety that specifically binds to the analyte. In a preferred embodiment, the capture agent is an antibody that specifically binds a protein of the protein profile.

Antibodies may be produced by any of a number of means well known to those of skill in the art (see, *e.g. Methods in Cell Biology Volume 37 Antibodies in Cell Biology,* Asai, ed. Academic Press, Inc. New York (1993); and *Basic and Clinical Immunology* 7th Edition, Stites & Terr, eds. (1991)). The antibody may be a whole antibody or an antibody fragment. It may be polyclonal or monoclonal, and it may be produced by challenging an organism (*e.g.* mouse, rat, rabbit. *etc.)* with a protein of the profile or an epitope derived therefrom. Alternatively, the antibody may be produced *de novo* using recombinant DNA methodology. The antibody can also be selected from a phage display library screened against the protein or interest (see, *e.g.* Vaughan *et al.* (1996) *Nature Biotechnology,* 14:309–314 and references therein).

Immunoassays also often utilize a labeling agent to specifically bind to and label the binding complex formed by the capture agent and the analyte, The labeling agent may

itself be one of the moieties comprising the antibody/analyte complex. Thus, the labeling agent may be a labeled profile protein or a labeled anti-profile protein antibody, Alternatively, the labeling agent may be a third moiety, such as another antibody, that specifically binds to the antibody/protein complex.

Other proteins capable of specifically binding immunoglobulin constant regions, such as protein A or protein G may also be used as the label agent. These proteins are normal constituents of the cell walls of streptococcal bacteria. They exhibit a strong non–immunogenic reactivity with immunoglobulin constant regions from a variety of species. *See*, generally Kronval, *et al. J. Immunol.;* 111:1401–1406 (1973), and Akerstrom, *et al., J. Immunol.*, 135:2589–2542 (1985).

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, analyte, volume of solution, concentrations, and the like. Usually, the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

1. Non–Competitive Assay Formats

Immunoassays for detecting proteins may be either competitive or noncompetitive. Noncompetitive immunoassays are assays in which the amount of captured analyte (in this case the protein profile) is directly measured. In one preferred "sandwich" assay, for example, the capture agent can be bound directly to a solid substrate where they are immobilized. These immobilized antibodies then capture the protein of interest present in the test sample. The protein thus immobilized is then bound by a labeling agent, such as a second antibody bearing a label. Alternatively, the second antibody may lack a label, but it may, in turn, be bound by a labeled third antibody specific to antibodies of the species from which the second antibody is derived. The second can be modified with a detectable moiety, such as biotin, to which a third labeled, molecule can specifically bind, such as enzyme–labeled streptavidin.

2. **Competitive Assay Formats**

In competitive assays, the amount of analyte (proteins of the protein profile) present in the sample is measured indirectly by measuring the amount of an added (exogenous) analyte displaced (or competed away) from a capture agent by the analyte present in the sample. In one competitive assay, a known amount of, in this case, the protein profile is added to the sample and the sample is then contacted with a capture agent. The amount of protein bound to the antibody is inversely proportional to the concentration of the protein present in the sample before the exogenous protein is added to the reaction mix.

In a particularly preferred embodiment, the antibody is immobilized on a solid substrate. The amount of protein bound to the antibody may be determined either by measuring the amount of protein present in an protein/antibody complex, or alternatively by measuring the amount of remaining uncompeted protein. The amount of protein may be detected by providing a labeled protein molecule.

A hapten inhibition assay is another preferred competitive assay. In this assay a known analyte, in this case the protein profile is immobilized on a solid substrate. A known amount of antibody is added to the sample, and the sample is then contacted with the immobilized protein. In this case, the amount of antibody bound to the immobilized protein is inversely proportional to the amount of protein present in the sample. Again the amount of immobilized antibody may be detected by detecting either the immobilized fraction of antibody or the fraction of the antibody that remains in solution. Detection may be direct where the antibody is labeled or indirect by the subsequent addition of a labeled moiety that specifically binds to the antibody as described above.

3. **Other Assay Formats**

In a preferred embodiment, Western blot (immunoblot) analysis is used to detect and quantify the presence of protein in the sample. The technique generally comprises separating sample proteins by gel electrophoresis on the basis of molecular weight, transferring the separated proteins to a suitable solid support, (such as a nitrocellulose filter, a nylon filter, or derivatized nylon filter), and incubating the sample with the antibodies that specifically bind protein. The anti–protein antibodies specifically bind to protein on the solid support. These antibodies may be directly labeled or alternatively may

be subsequently detected using labeled antibodies (*e.g.*, labeled sheep anti–mouse antibodies) that specifically bind to the anti-protein. A particularly preferred protocol for Western Blot detection of human or animal protein in CSF is provided in Example 1.

Other assay formats include liposome immunoassays (LIA), which use liposomes designed to bind specific molecules (*e.g.*, antibodies) and release encapsulated reagents or markers. The released chemicals are then detected according to standard techniques *(see, Monroe et at.* (1986) *Amer. Clin. Prod. Rev.* 5:34–41).

4. Labels

The particular label or detectable group used in the assay is not a critical aspect of the invention, so long as it does not significantly interfere with the specific binding of the antibody used in the assay. The detectable group can be any material having a detectable physical or chemical property. Such detectable labels have been well developed in the field of immunoassays and, in general, most any label useful in such methods can be applied to the present invention. Thus, a label is any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or chemical means. Useful labels in the present invention include magnetic beads (*e.g.* Dynabeads™), fluorescent dyes (*e.g.*, fluorescein isothiocyanate, texas red, rhodamine, and the like), radiolabels (*e.g.*, ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (*e.g.*, horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (*e.g.* polystyrene, polypropylene, latex, *etc.)* beads.

The label may be coupled directly or indirectly to the desired component of the assay according to methods well known in the art. As indicated above, a wide variety of labels may be used, with the choice of label depending on sensitivity required, ease of conjugation with the compound, stability requirements, available instrumentation, and disposal provisions.

Non–radioactive labels are often attached by indirect means. Generally, a ligand molecule (*e.g.*, biotin) is covalently bound to the molecule. The ligand then binds to an anti–ligand (*e.g.*, streptavidin) molecule which is either inherently detectable or covalently bound to a signal system, such as a detectable enzyme, a fluorescent compound, or a chemiluminescent compound. A number of ligands and anti–ligands can be used. Where

a ligand has a natural anti–ligand, for example, biotin, thyroxine, and cortisol, it can be used in conjunction with the labeled, naturally occurring anti–ligands. Alternatively, any haptenic or antigenic compound can be used in combination with an antibody.

The molecules can also be conjugated directly to signal generating compounds, *e.g.*, by conjugation with an enzyme or fluorophore. Enzymes of interest as labels will primarily be hydrolases, particularly phosphatases, esterases and glycosidases, or oxidoreductases, particularly peroxidases. Fluorescent compounds include fluorescein and its derivatives, rhodamine and its derivatives, dansyl, umbelliferone, etc. Chemiluminescent compounds include luciferin, and 2,3–dihydrophtlialazinediones, *e.g.*, luminol. (For a review of various labeling or signal producing systems which may be used, *see*, U.S. Patent No. 4,391,904).

Means of detecting labels are well known to those of skill in the art. Thus, for example, where the label is a radioactive label, means for detection include a scintillation counter or photographic film as in autoradiography. Where the label is a fluorescent label, it may be detected by exciting the fluorophore with the appropriate wavelength of light and detecting the resulting fluorescence. The fluorescence may be detected visually, by means of photographic film, by the use of electronic detectors such as charge coupled devices (CCDs) or photomultipliers and the like. Similarly, enzymatic labels may be detected by providing the appropriate substrates for the enzyme and detecting the resulting reaction product. Finally simple colorimetric labels may be detected simply by observing the color associated with the label. Thus, in various dipstick assays, conjugated gold often appears pink, while various conjugated beads appear the color of the bead.

Some assay formats do not require the use of labeled components. For instance, agglutination assays can be used to detect the presence of the target antibodies. In this case, antigen–coated particles are agglutinated by samples comprising the target antibodies. In this format, none of the components need be labeled and the presence of the target antibody is detected by simple visual inspection.

D) Diagnostic Imaging

One or more of the proteins making up the disease protein profiles obtained using the methods described herein can also be used as markers in diagnostic imaging techniques, for example magnetic resonance imaging (MRI).

III. Determining Disease Severity Using the Protein Profile

One or more the proteins which make up the protein profiles as described herein can also be useful in determining disease severity. In particular, an indicator of disease severity can be the absolute or relative amount by which one or more proteins of the profile have increased or decreased as compared to healthy control levels. For example, as shown in Figure 4, panels A through C, severity of PD is correlated to decrease in CIT-5 (Apo-A1 lipoprotein). Panel A shows that the relative quantity of Apo-A1 lipoprotein decrease as the "H+Y" rating of disease severity increases. The least severely affected patients (shown as "1") have more Apo-A1 lipoprotein than the more severely affected patients (shown as "2.5" or "3"). Panel B shows that a decrease in Apo-A1 lipoprotein also correlates to age and duration of PD. Young patients with short disease duration ("YS") have relatively more Apo-A1 lipoprotein than young patients with long duration ("YL") or old patients with either short ("OS") or long ("OL") duration disease. Panel C expresses Apo-A1 lipoprotein amounts plotted against clinical parameters. A rating of "1" indicates short duration PD with no dyskinesia, and no or minimum dopamine therapy. A "2" indicates medium duration disease, dyskinesia and low dopaminergic therapy. A "3" indicates long duration, dyskinesia and moderate to high dopaminergic therapy. As the rating increases, the relative amount of Apo-A1 lipoprotein decreases.

IV. Therapeutic Monitoring and Patient Management using Disease-Specific Protein Profiles

The disease specific protein profiles which are identified as described herein are also useful in therapeutic monitoring and patient management. For example, using the schizophrenia protein profile described herein, changes in the levels of the individual proteins in response to drug treatment can be evaluated. As shown in Figures 7 to 10, when the protein profile of untreated schizophrenics is compared to those on some conventional treatment regimes, differences in the levels of the individual proteins is observed. In some cases, the treatments drive the protein level closer to normal (Figures 8 and 10). In this way, it is contemplated that new treatment regimes could be developed by examining the levels of the protein profile. Similarly, the protein profiles of individual subjects could be monitored for example, to determine progression of the disease. The

present invention, therefore, provide a rapid and efficient way to monitor treatment regimes and to determine if an individual is deteriorating or improving.

The protein profiles are also useful in developing drugs using animal models of disease. Human disease models can be created in laboratory animals, for instance, by inserting a human gene(s) into the animal. The protein profiles obtained by the methods described herein are applicable to these animal models and can be used to monitor treatment protocols and regimes in these animal models.

The following examples are provided to illustrate but not limit the present invention.

EXAMPLES

Example 1: Preparation of Samples

Cerebrospinal fluid samples were kindly provided by various neurologists and veterinarians. The diagnoses were made by the referring physicians according to standard clinical criteria in addition to pathological studies as appropriate. The CSF was collected and the samples were immediately frozen at -70°C before shipment to our laboratory. Upon receipt, samples were thawed and aliquoted for use. Samples for 2-DE were prepared by the addition of 9M urea, 2% 2-mercaptoethanol, 2% NP-40, 0.8% BioLyte pH 3-10 carrier ampholytes, and 0.002% Bromophenol blue. For SDS-PAGE, 50 µL CSF was added to 20 µL Laemmli sample buffer, heated for 5 minutes at 95°C, and loaded.

Two-Dimensional Electrophoresis (2DE)

Samples were separated by two-dimensional electrophoresis and silver stained as previously described Harrington *et al.* (1991) *Methods: A Companion to Methods in Enzymology* 3:135-141. Two dimensional electrophoresis consisted of isoelectric focusing followed by electrophoresis using a polyacrylamide gel. Isoelectric focusing was done in immobilized pH gradients. The 180 mM linear pH 3-10 gradient (Pharmacia, LKB, Piscataway, New Jersey, USA, catalog #80-1128-30) were used. These gradients were rehydrated, electrophoresed, and equilibrated as described in Bjellqvist *et al.* (1993) *Electrophoresis* 14:1375-1378 and Bjellqvist *et al.* (1993) *Electrophoresis* 14:1357-1365. The second dimension was performed in 14% T polyacrylamide gels and silver stained as

described in Harrington *et al.* (1991) *Methods: A Companion to Methods in Enzymology* 3:98-108 or electroblotted as described in Towbin *et al.* (1979) *Proc. Nat'l. Acad. Sci.* 76:4350-4354 onto nitrocellulose (Schleicher & Schuell, catalog # 0830N) or PVDF (Millipore, Massachusetts, USA). The blots were stained with Coomassie Brilliant Blue R-250 or silver stained.

Evaluation of two dimensional gels

Two-dimensional gel electrophoresis gels and blots were analyzed either by direct visual comparison, by comigration studies, and/or with the aid of computer image analysis software. GALtool (Solomon *et al.* (1993) *CABIOS* 9:133-139") or Melanie (BioRad). The raw data file for each sample is then processed by computer-assisted algorithms. The algorithm was performed as follows. First, the protein spots were identified using an edge detector or peak identifier. The x and y co-ordinates corresponding to pH and molecular weight along with total integrated density were recorded. Next, all spots were compared from one sample to another. A master gel was used to retain all the data.

The compared gel data was then split into groups of each disease and normal controls. Similarities and differences were noted. All of the different proteins were then further analyzed in subsets of patients within each disease compared to controls.

The above-described steps were subjected to multi-dimensional discriminant function analysis using either BMDP™ 7M Mainframe programs (BMDP Statistical Solutions Ltd., Cork, Ireland) or Unistat programs (Unistat House, London, England). Linear and nonlinear discriminant function analyses were performed. All variable proteins were examined for combinations that would provide the best discrimination and then the best discrimination were investigated. Once these were determined, these proteins were used to test a second, new population of samples. Only when these same proteins gave discriminating power in the second population was this group of proteins considered for the profile.

Proteins altered in neurologic disorders that may not uniquely discriminate the disease by themselves are studied to identify whether in combination a number of proteins can be form a profile which is specific for neurologic disorders as described in Example 2. The proteins can be identified by their migration in the gel. In addition, each protein in the profile can be identified by obtaining a molecular footprinting, for example, by one or

more of the following: antibody assay, protein sequence determination, mass spectrometry and database comparison.

Example 2: Preparation of Specific Protein Profiles

1. Alzheimer's Disease (AD)

Six samples from AD patients were used in the computation described in Example 1. The profile was composed of 18 proteins, or spots, designated AD-1 through AD-18 which were altered in presence or amount from four healthy controls. All 18 spots present in the AD samples were not present in healthy controls. AD-1 through AD-18 are identified by name or by approximate molecular weight and isoelectric point in Table 1. Another profile specific for AD has been identified using a combination of proteins that include 2 of those qualitative markers with 7 other proteins that are altered in various diseases.

2. Parkinson's Disease (PD)

Six samples from PD patients were used to determine a protein profile as described above. Eight proteins were found to be qualitatively or quantitatively altered in PD samples when compared to normal controls. The protein spots are identified in Table 1. CIT-A, CIT-B and CIT-C were present in PD samples and were not present in healthy controls. CIT-1 (alpha 1 microglobulin), CIT-3 (PGDS isoform), CIT-4 (complement 4 gamma) and CIT-5 (Apo-A1 lipoprotein) were decreased in PD samples as compared to controls, while CIT-2 (PGDS isoform) was increased in PD samples.

3. Schizophrenia

Twenty samples from schizophrenic patients were used in the computation described in Example 1. The profile was composed of seven proteins, or spots, which were altered in presence or amount from 100 healthy controls matched for age and sex and 70 disease controls having disorders including manic depression, CJD, viral encephalitis, subacute sclerosing panencephalitis, Korsakoff's psychosis, benign essential tremor, Huntington's disease, cerebrovascular disease, Alzheimer's disease, motor neuron disease, bacterial meningitis, systemic lupus erythematosis and headache. Results are shown in Tables 2 and 3.

Table 2: Schizophrenia

Protein identification	Concentration (relative to normal levels)
protein 91	0.54
protein 48	1.27
orosomucoid	0.63
prostaglandin D synthetase (PGDS)	0.8
antithrombin III	0.7
protein 46	1.23

Table 3

Protein identification	Qualitative Changes (in percentage of patient population)
fibrinogen degradation products	30%

As these results indicate, a protein profile specific for schizophrenia is characterized by decreased amounts of PGDS, orosomucoid, antithrombin III and protein 91 as well as increased amounts of protein 48 and protein 46 when compared to normal CSF levels. Fibrinogen degradation products are absent in normal controls and appear in samples obtained from schizophrenics.

4. Multiple Sclerosis

Twenty samples from multiple sclerosis patients were used in the computation described in Example 1. One hundred age and sex matched controls were used along with 70 other disorders including manic depression, CJD, viral encephalitis, subacute sclerosing panencephalitis, Korsakoff's psychosis, benign essential tremor, Huntington's disease, cerebrovascular disease, Alzheimer's disease, motor neuron disease, bacterial meningitis, systemic lupus erythematosis and headache. Using the methods described above, a protein profile for multiple sclerosis was obtained and is shown in Table 4 and 5.

Table 4: Multiple Sclerosis

Protein identification	Concentration (relative to normal levels)
ApoD (protein 108)	1.56
ApoD (protein 110)	2
proteins 91 and 92	2
α_1 β-glycoprotein	2

Apo J/K	1.6
transthyretin	1.4
C4γ	2.5
Ig K and L chains	2

Table 5: Multiple Sclerosis

Protein identification	**Qualitative Changes (in percentage of patient population)**
fibrinogen degradation products	13%

The protein profile for multiple sclerosis includes ApoD (protein 108), ApoD (protein 110), protein 91, protein 92, α_1 β-glycoprotein, ApoJ/K, transthyretin, C4γ, and Ig K and L chains. In affected individuals levels of these proteins are elevated as compared to normal controls. Fibrinogen degradation products also appear in these subjects.

5. Creutzfeldt-Jakob Disease (CJD)

Twenty samples of CJD were used to compute a protein profile. The samples were matched by age and sex to 100 normal controls and to 70 disease controls having disorders including manic depression, multiple sclerosis, viral encephalitis, subacute sclerosing panencephalitis, Korsakoff's psychosis, benign essential tremor, Huntington's disease, cerebrovascular disease, Alzheimer's disease, motor neuron disease, bacterial meningitis, systemic lupus erythematosis and headache. As previously discussed, 99% of CJD patients show the appearance of 14-3-3 protein when compared to normal controls. In addition, 67% show the appearance of fibrinogen degradation products.

6. Subacute Sclerosing Panencephalitis (SSPE)

Using 8 samples in the methods described above, a protein profile composed of proteins APOA1 isoforms, APOJ isoforms, isoforms of haptoglobin β-chain, 46, GC globulin, 91, C3-activator and Ig kappa and lambda chains was determined for SSPE. Figure 5 where the multidimensional discriminating space is plotted in 2-dimensions and illustrates the diagnosis of subacute sclerosing panencephalitis (SSPE) patients in Group 5, individually labeled E with complete resolution from the other patient groups. These results indicate that the present method can distinguish between SSPE and multiple sclerosis, two distinct disorders which cannot be differentiated using standard biochemical analyses.

Example 3: Response of Schizophrenia Protein Profile Markers to Drug Treatment

The seven protein markers which make up the protein profile identified in Example 2 were analyzed to determine if their levels were affected by drug treatment. Protein quantity of each marker from normal controls (normal), untreated schizophrenics (none), and schizophrenics taking fluphenazine and chloropromazine (Flu + CPZ) or fluphenazine and lithium (Flu + Li) or fluphenazine alone (Flu). As shown in Figures 2 and 4, levels of protein 48 and protein 46 in schizophrenics taking drugs is closer to normal levels than in untreated patients. Interestingly, as shown in Figure 1, protein 91, which is decreased in the CSF of schizophrenia patients, is further decreased in schizophrenic patients who are following drug treatment.

PGDS levels are decreased in both treated and untreated schizophrenic patients, however, in patients taking fluphenazine alone or fluphenazine and lithium, the levels of PGDS are closer to normal controls. (Figure 3). The inventor has previously shown that altered levels of PGDS glycoforms are correlated with symptoms of sleep disorders. The present invention also indicates how PGDS levels correlate with sleep disorders. The schizophrenic patients who were on treatment regimes in which PGDS levels were closer to normal also had fewer sleep related symptoms. Thus, the present invention provides a method of monitoring treatment regimes and correlating protein levels to symptoms.

CLAIMS

1. A method of diagnosing a neurological disorder in a biological sample, the method comprising:

(a) obtaining a disease-specific protein profile by detecting proteins whose levels are altered in a subject having a neurological disorder when compared to levels of a healthy control;

(b) obtaining the biological sample;

(c) measuring the levels of the disease-specific protein profile obtained in step (a) in the biological sample; and

(d) comparing the levels of the disease-specific protein profile in the biological sample to levels found in the healthy control;

wherein altered levels of the protein profile of the biological sample as compared to the healthy control is diagnostic of the neurological disorder.

2. The method according to claim 1, wherein the biological sample is cerebrospinal fluid.

3. The method according to claim 1, wherein the neurologic disorder is selected from the group consisting of schizophrenia, Parkinson's disease (PD) and Alzheimer's Disease (AD).

4. The method according to claim 1, wherein the disease-specific protein profile is obtained using 2 dimensional gel electrophoresis (2DE).

5. The method according to claim 1, wherein the disease-specific protein profile is obtained by Western blotting.

6. The method according to claim 1, wherein the disease-specific protein profile is obtained by immunoassay.

7. The method according to claim 1, wherein in step (c) the protein profile is measured by visualizing a 2DE gel.

8. The method according to claim 1, wherein in step (c) the protein profile is measured using immunoassay.

9. The method according to claim 1, wherein in step (c) the protein profile is measured using Western blotting.

10. The method according to claim 1 wherein the neurologic disorder is Alzheimer's Disease (AD).

11. The method according to claim 10 wherein the protein profile comprises proteins AD1 through AD18.

12. The method according to claim 11, wherein proteins AD1 through AD18 are present or increased in samples obtained from AD patients as compared to samples obtained from healthy controls.

13. The method according to claim 1 wherein the neurologic disorder is Parkinson's disease.

14. The method according to claim 13 wherein the protein profile comprises CIT-A, CIT-B, CIT-C (protein 129), CIT-1 (alpha 1 microglobulin subunit), CIT-2 (PGDS isoform), CIT-3 (PGDS isoform), CIT-4 (complement 4 gamma) and CIT-5 (Apo-A1 lipoprotein).

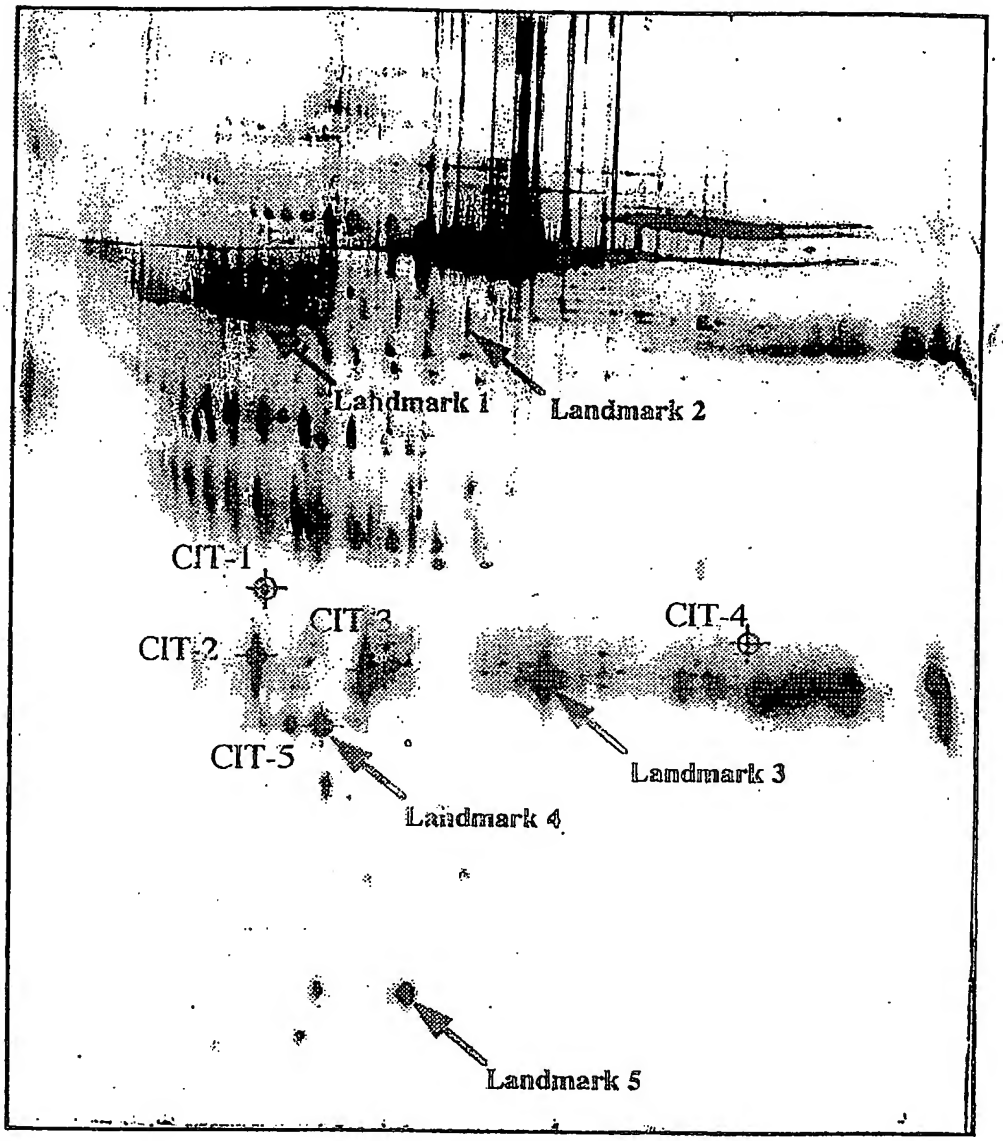
15. The method according to claim 14 wherein CIT-A, CIT-B and CIT-C are present in PD patients and absent in healthy controls, CIT-2 is increased in PD patients as compared to healthy controls and CIT-1, CIT-3, CIT-4 and CIT-5 are decreased in PD patients as compared to healthy controls.

16. The method according to claim 1 wherein the neurologic disorder is multiple sclerosis.

17. The method according to claim 15 wherein the protein profile comprises ApoD (protein 108), ApoD (protein 110), protein 91, protein 92, α_1 β-glycoprotein, ApoJ/K, transthyretin, C4γ, and Ig K and L chains.

18. The method according to claim 1 wherein the neurologic disorder is subacute sclerosing panencephalitis (SSPE).

19. The method according to claim 17 wherein the protein profile comprises APOA1 isoforms, APOJ isoforms, isoforms of haptoglobin β-chain, 46, GC globulin, 91, C3-activator and Ig kappa and lambda chains.


Landmark 1
Landmark 2
CIT-1
CIT-2
CIT-3
CIT-4
CIT-5
Landmark 3
Landmark 4
Landmark 5

Normal

FIG 1

Parkinson's Disease

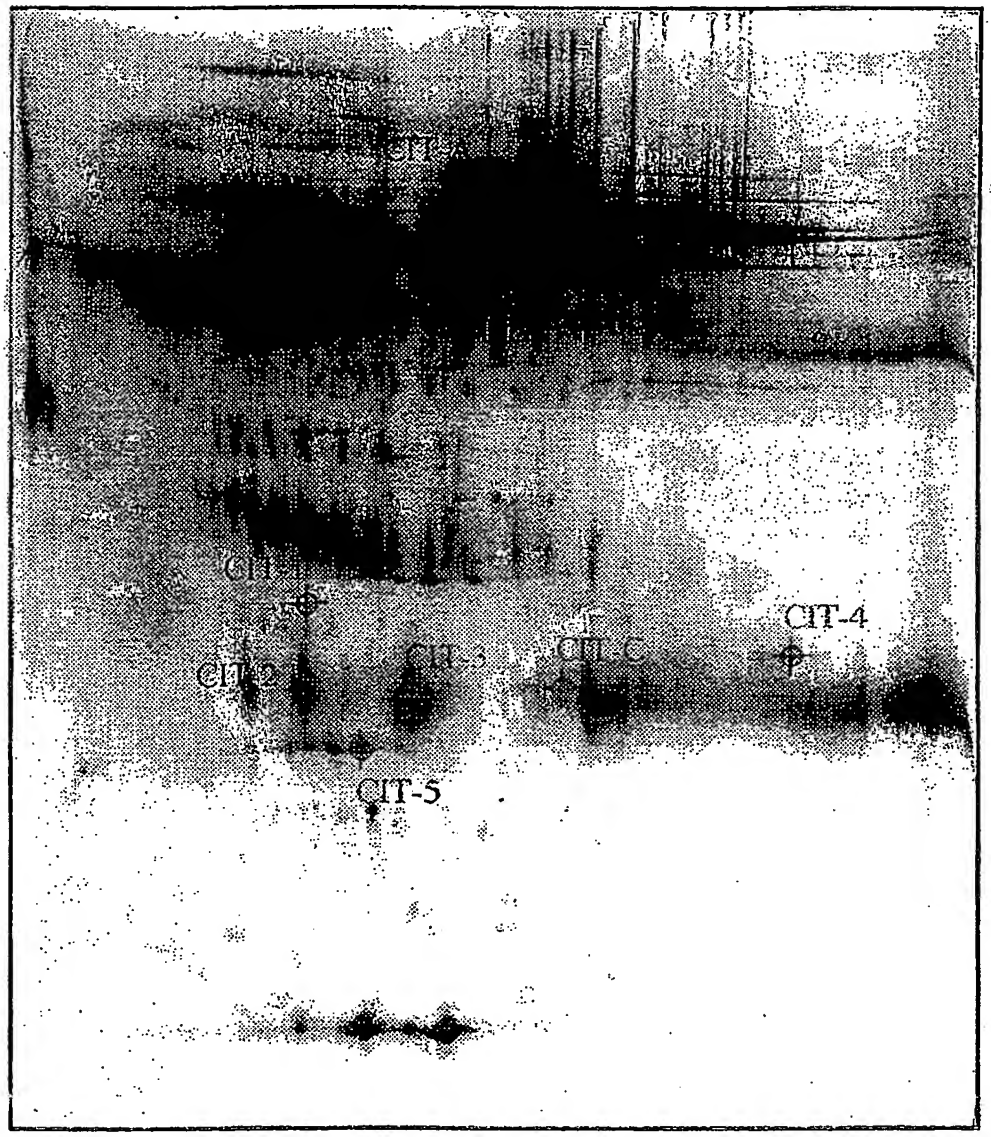
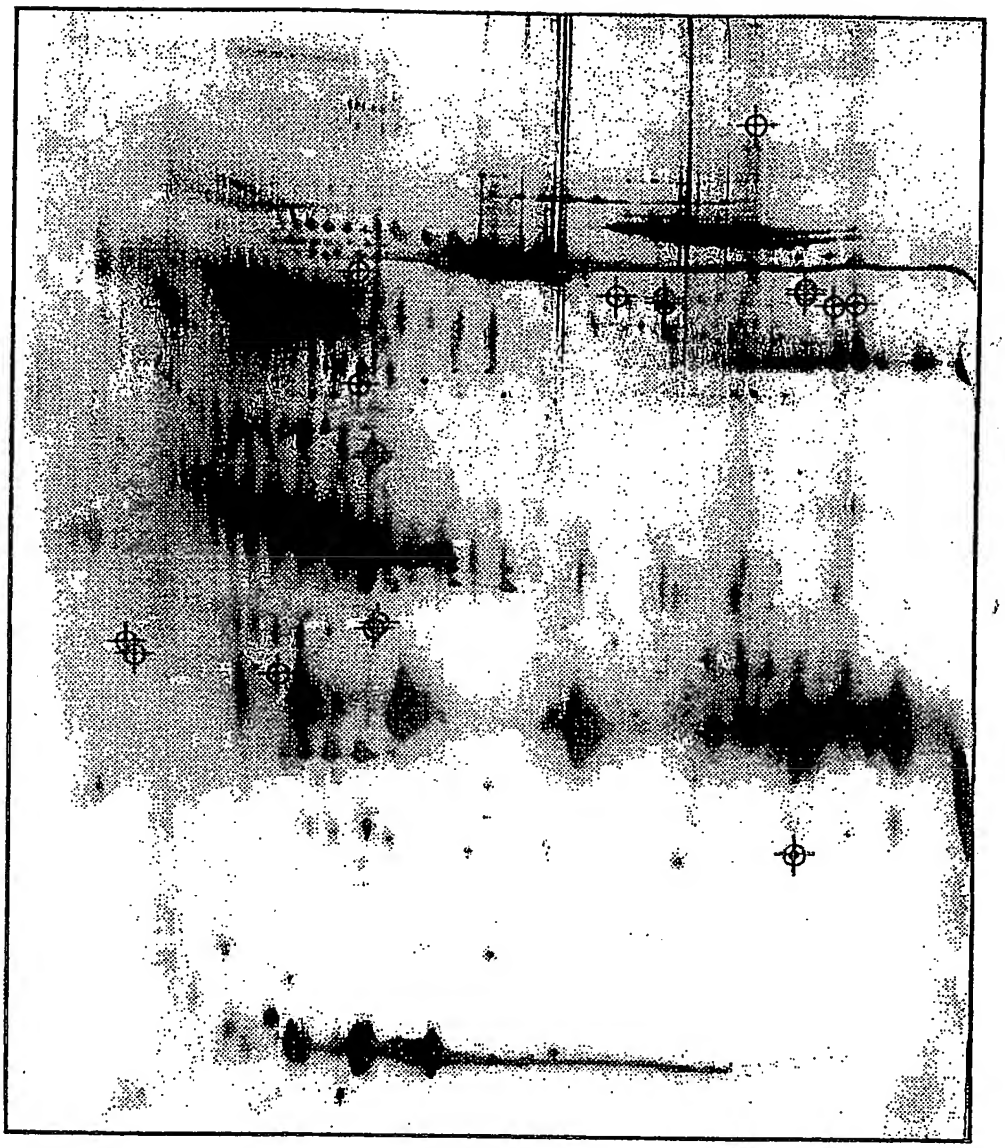


FIG 2



Alzheimer's Disease

FIG 3

Fig. 4A

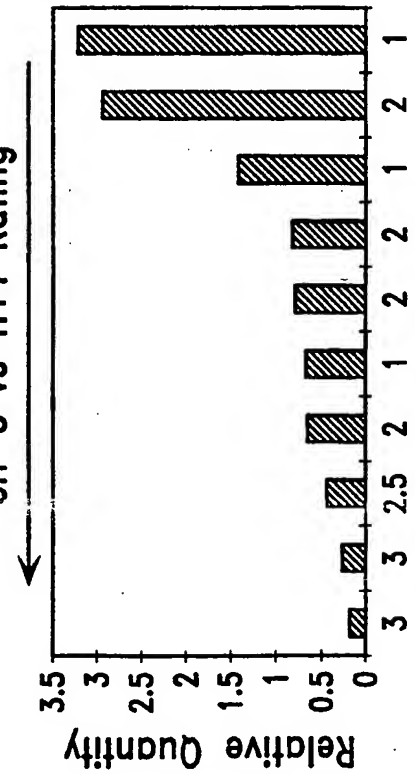


Fig. 4B

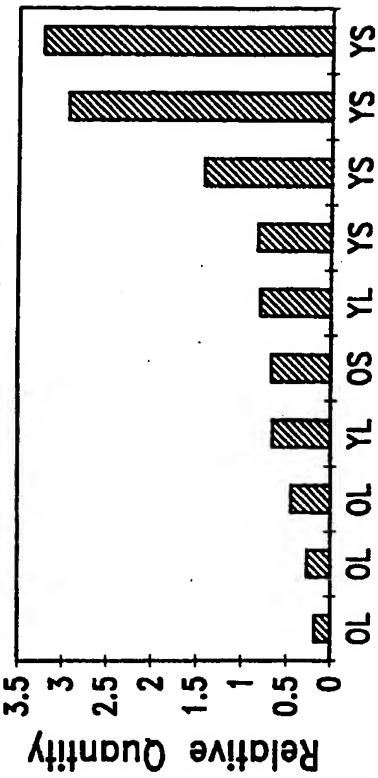
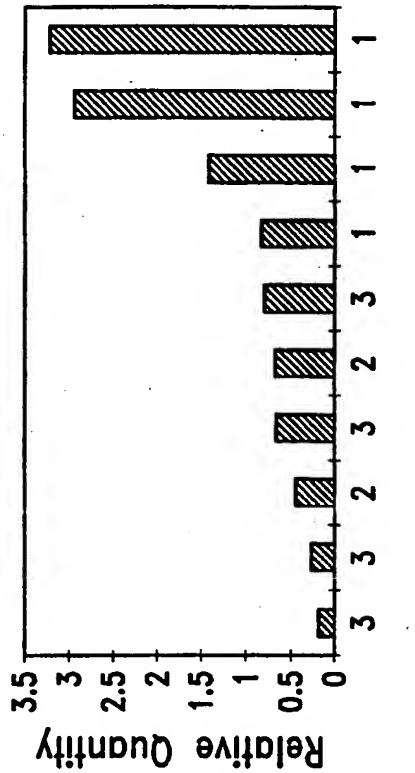


Fig. 4C



1-Short duration, no dyskinesia, no/minimum dopaminergic therapy

2-Medium duration, dyskinesia, low dopaminergic therapy

3-Long duration, dyskinesia, high/moderate dopaminergic therapy

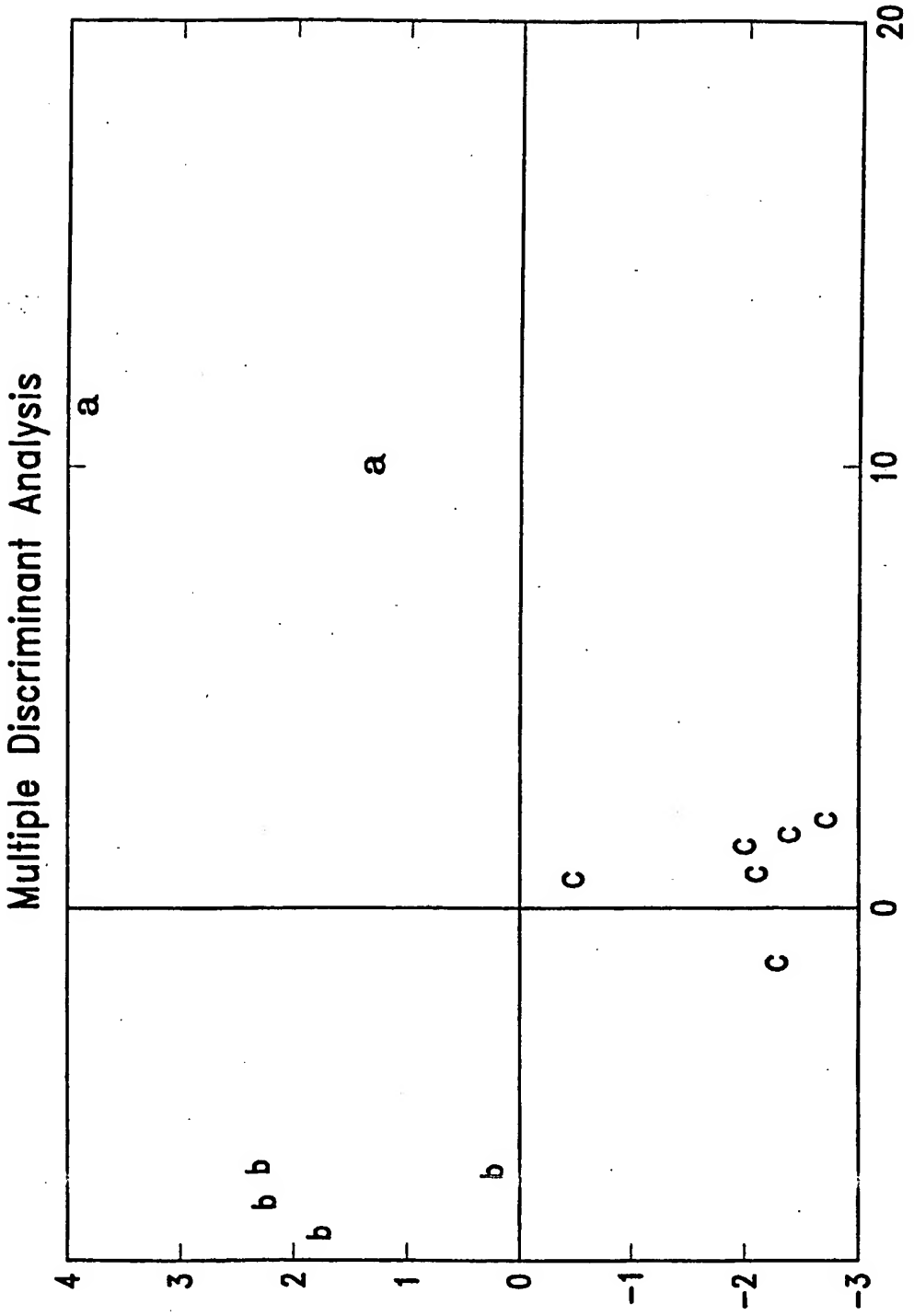

Multiple Discriminant Analysis
a
a
b b
b
b
c
c c
c
c
c
20
10
0
4
3
2
1
0
-1
-2
-3

Fig. 5

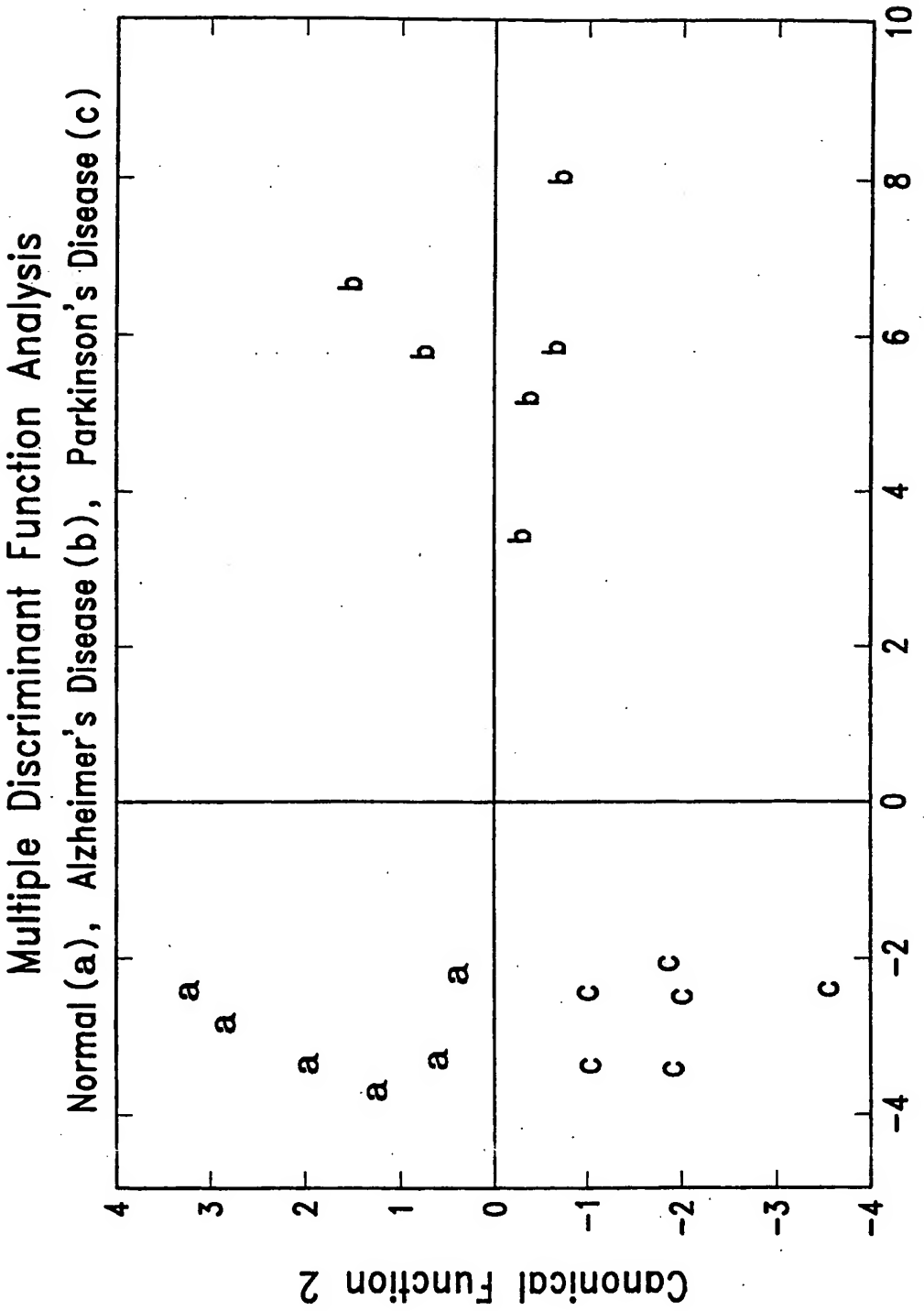

Multiple Discriminant Function Analysis
Normal (a), Alzheimer's Disease (b), Parkinson's Disease (c)
Canonical Function 2
Canonical Function 1
4
3
2
1
0
-1
-2
-3
-4
10
8
6
4
2
0
-2
-4
a
b
c

Fig. 6

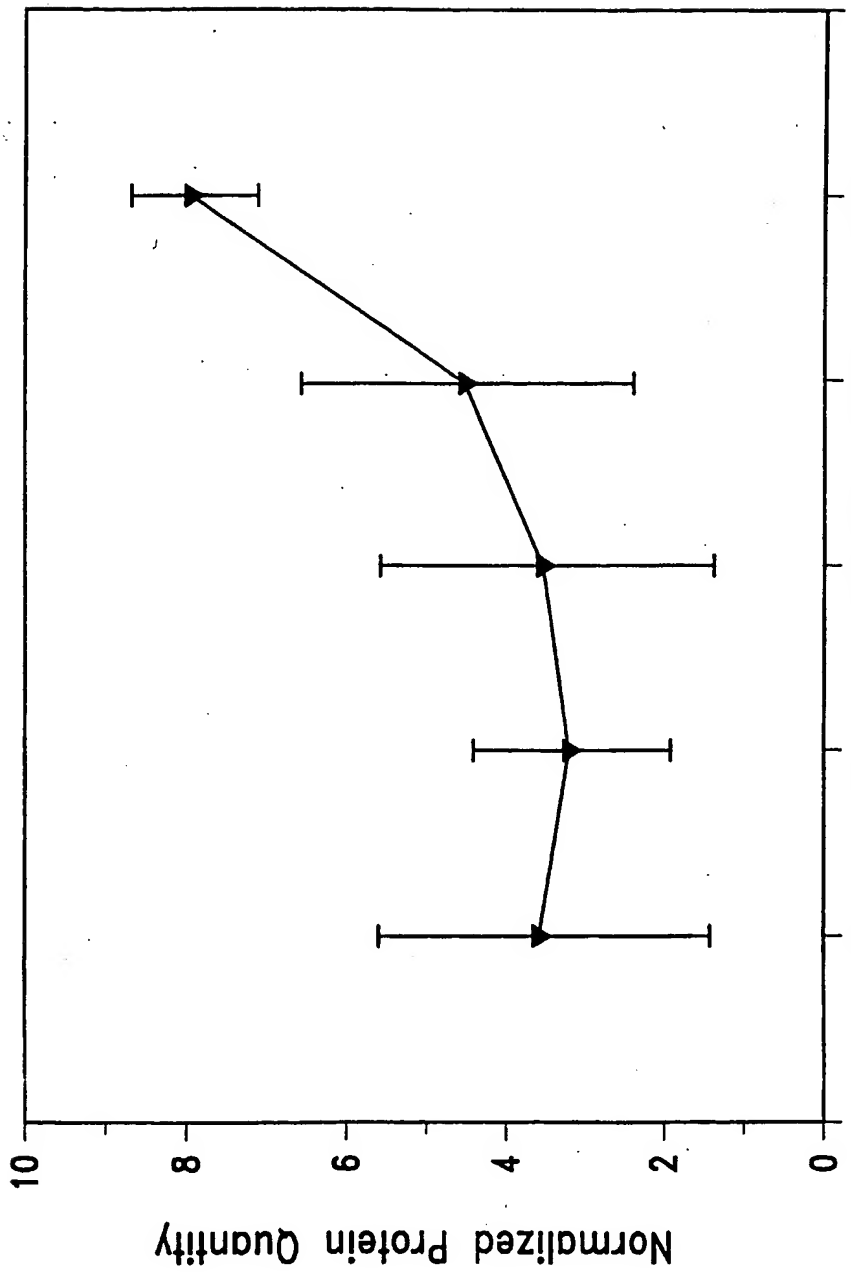

Normalized Protein Quantity
0
2
4
6
8
10
Flu
Flu+Li
Flu+CPZ
None
Normal
Drug Treatment
95% Confidence levels

Fig. 7

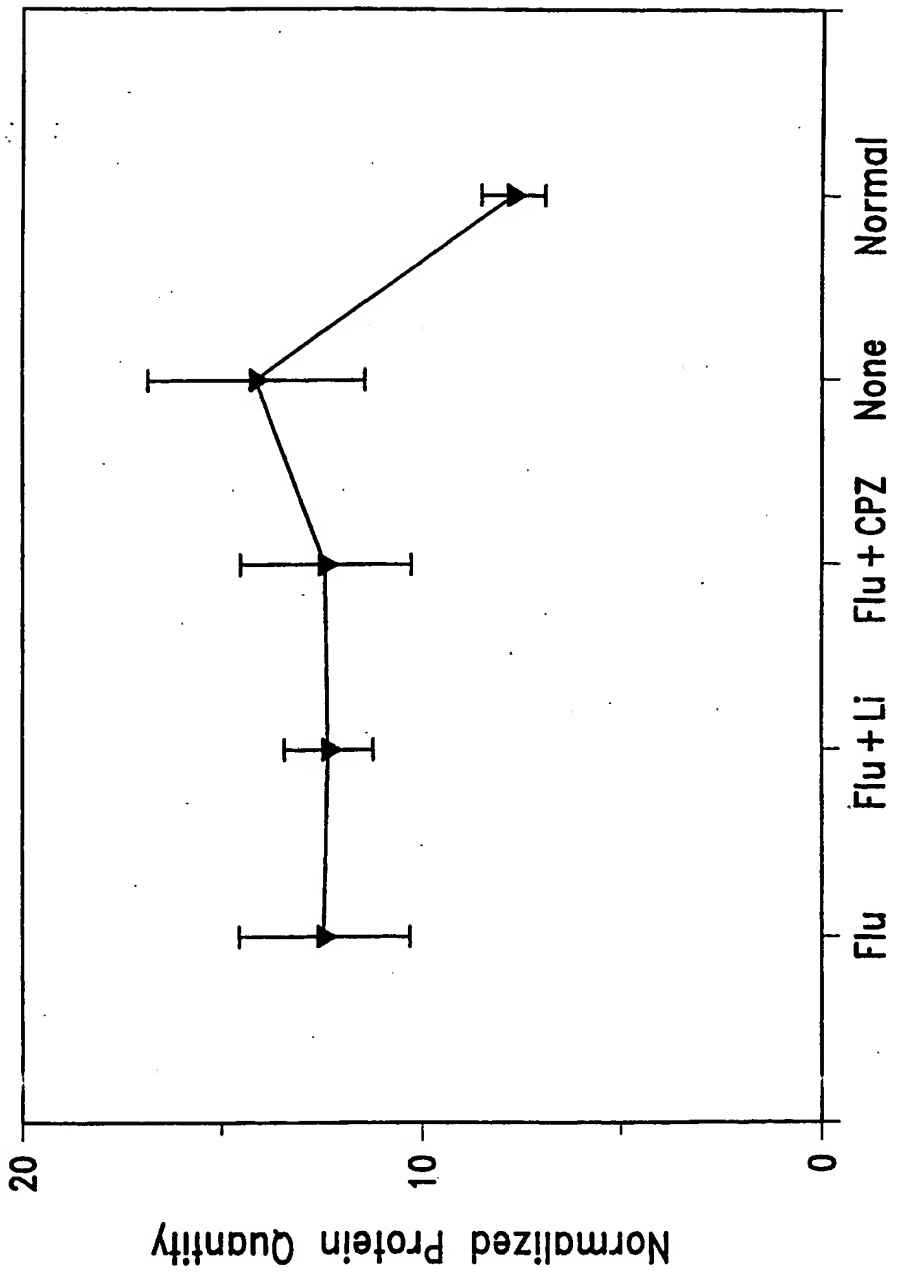

Normalized Protein Quantity
20
10
0
Flu
Flu + Li
Flu + CPZ
None
Normal
Drug Treatment
95% Confidence limits

Fig. 8

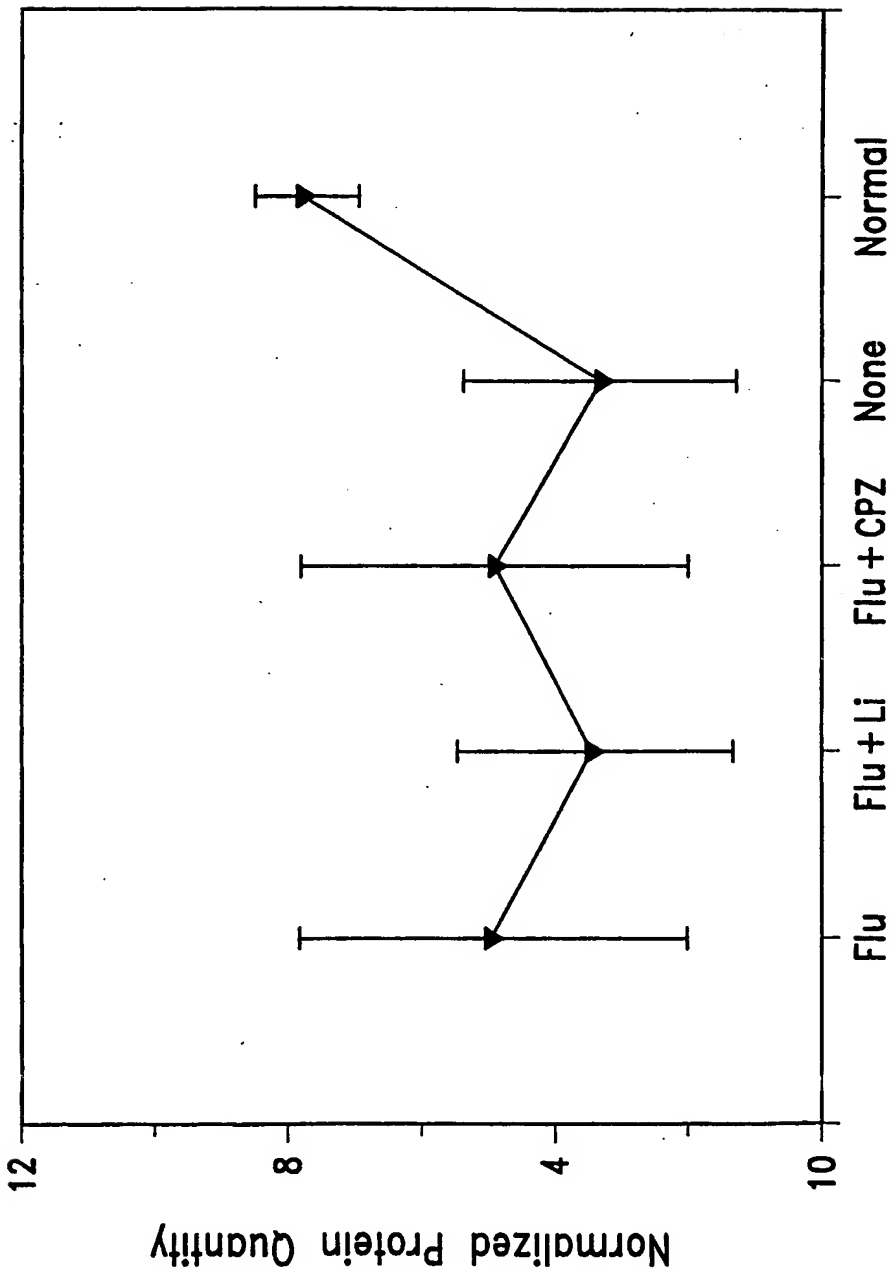

Normalized Protein Quantity
12
8
4
10
Flu
Flu+Li
Flu+CPZ
None
Normal
Drug Treatment
95% Confidence

Fig. 9

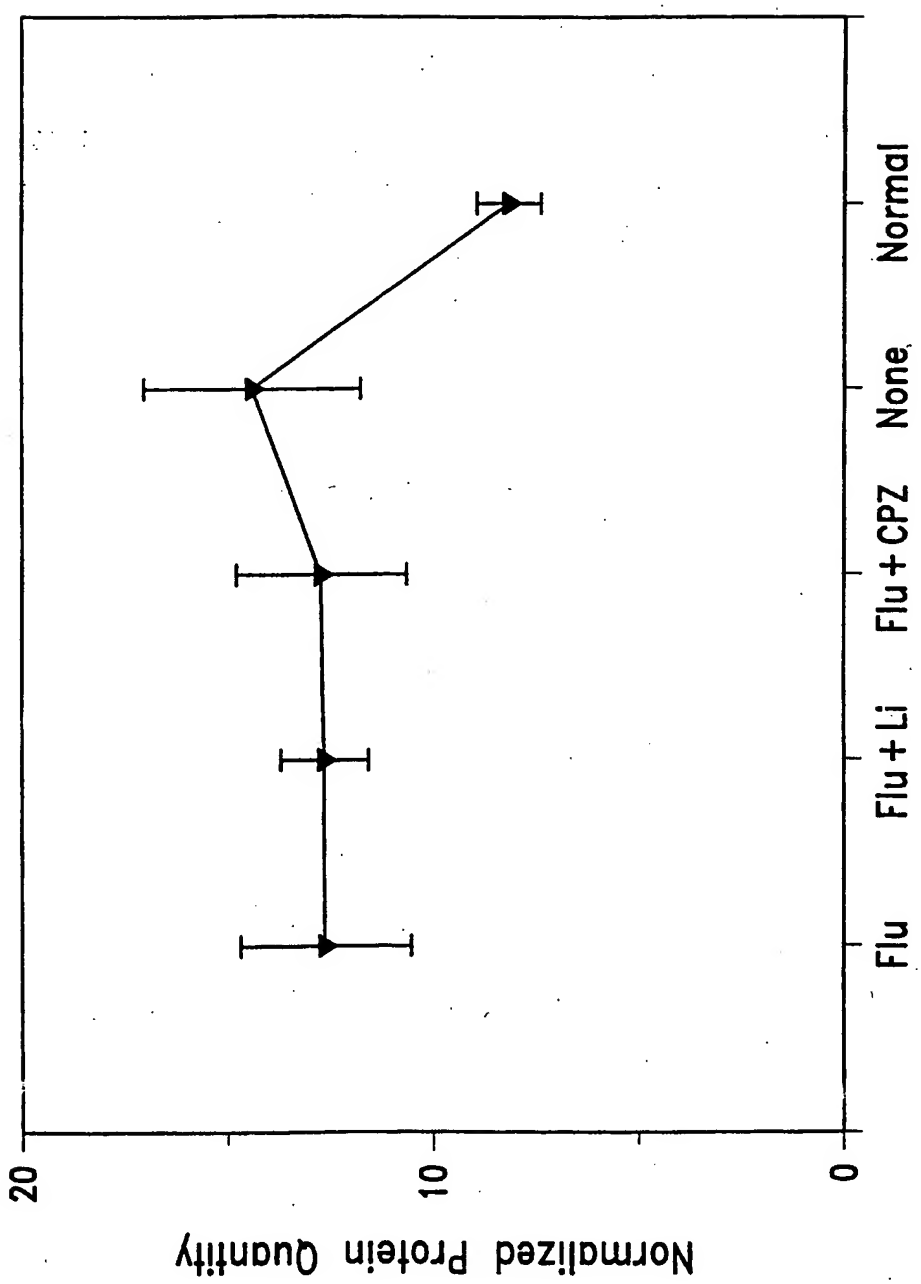

Normalized Protein Quantity
20
10
0
Flu
Flu + Li
Flu + CPZ
None
Normal
Drug Treatment
95% Confidence limits

Fig. 10

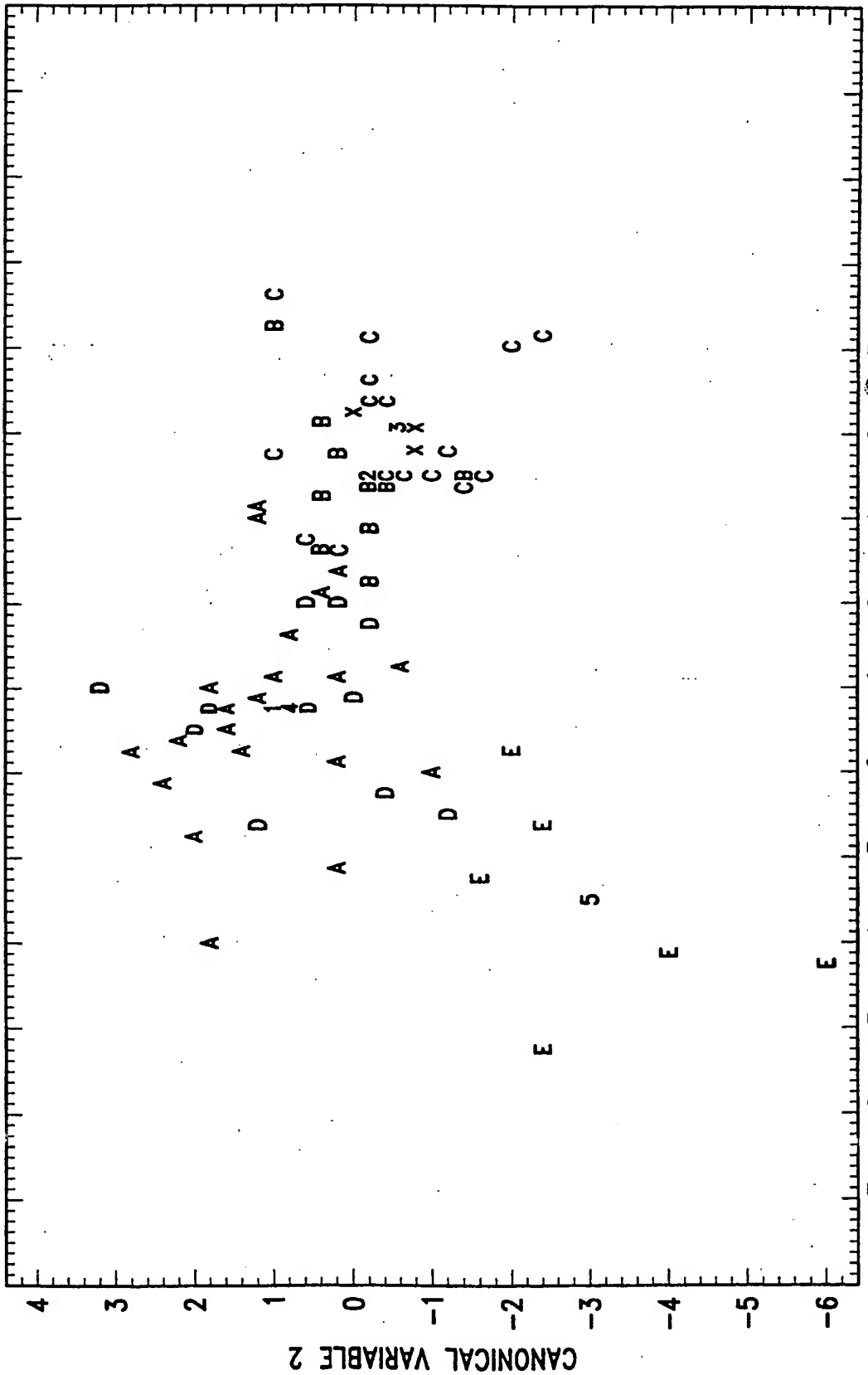


FIG. 11

INTERNATIONAL SEARCH REPORT

In ..ational Application No
PCT/US 98/05045

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68

According to International Patent Classification(IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 33992 A (UNIV BAR ILAN ;MOR RESEARCH APPLIC LTD (IL); SHALIT FRANCES (IL)) 14 December 1995 see claims see page 4, line 9 - page 5, line 2 see page 6, line 23 - page 8, line 4 see page 9, line 19 - line 25	1-19
X	WO 95 05604 A (MOLECULAR GERIATRICS CORP ;US GOVERNMENT (US)) 23 February 1995 cited in the application see claims 1,2,8,9 see page 3, line 6-10 see page 7, line 1 - line 13 see page 12, line 26 - page 13, line 23	1-19

-/--

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 July 1998	14/08/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Routledge, B

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INTERNATIONAL SEARCH REPORT

In ..national Application No
PCT/US 98/05045

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92 13273 A (ESA INC) 6 August 1992 see claims 1-8 see page 8, line 1 - line 8	1-19
X	US 4 874 694 A (GANDY SAMUEL E ET AL) 17 October 1989 cited in the application see claims see column 2, line 67 - column 3, line 27	1-19
X	WO 86 03006 A (ESA INC) 22 May 1986 see claims 28-31 see page 15, line 6 - page 16, line 3	1-19
X	BEHAN ET AL: "Serum Proteins, amyloid and Alzheimer's disease" J.AMERICAN GERIATRICS SOC., vol. 18, no. 10, - October 1970 pages 729-797, XP002070711 see table 1 see page 796	1-19

1

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Int tional Application No
PCT/US 98/05045

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		EP 0760098 A	05-03-1997
WO 9505604 A	23-02-1995	AU 7556994 A	14-03-1995
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		EP 0567564 A	03-11-1993
		JP 6504623 T	26-05-1994
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		DE 3587091 A	25-03-1993
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		JP 62500805 T	02-04-1987
		US 4863873 A	05-09-1989

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

Invention 1: 1-39 (partially)

Method for diagnosis of multiple sclerosis using the feature/isoform MSF-1/MSPI-1 as marker; antibodies against said marker and a kit comprising said antibodies; pharmaceutical composition comprising said marker or said antibodies; A method for screening for agents that interact with said marker; Use of said antibody, said marker or said modulating agent for treating multiple sclerosis.

Inventions 2-311: 1-39 (partially)

Method for diagnosis of multiple sclerosis using the features/isoforms MSF-2 to MSF-325/MSPI-2 to 325.2 as markers; antibodies against said markers and a kit comprising said antibodies; pharmaceutical composition comprising said markers or said antibodies; A method for screening for agents that interact with said markers; Use of said antibodies, said markers or said modulating agents for treating multiple sclerosis.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 02/00330

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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		CA 2321160 A1	19-08-1999
		CN 1295668 T	16-05-2001
		EP 1055126 A1	29-11-2000
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		PL 342365 A1	04-06-2001
		ZA 9901184 A	15-08-2000